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THE SEQUENCE OF OSSIFICATION OF THE HUMAN VERTEBRAL NEURAL ARCH CENTRES

by

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ABSTRACT

Textbook descriptions regarding the pattern of ossification of the developing spine in the human fetus suggest that ossification of the vertebral neural arches occurs in a cranio-caudal direction similar to the formation of somites. Recent radiographic evidence (Bagnall 1976) suggests that ossification of the neural arches first occurs in the lower cervical/upper thoracic regions quickly followed by a second group in the upper cervical region and that a third group develops subsequently in the lower thoracic/upper lumbar region.

With these differing viewpoints in mind, ten unsexed, formalin-fixed and fresh-frozen human embryos and fetuses, eighteen fresh albino rat fetuses and 108 human specimens which had been serial sectioned were examined. Crown-rump length (CRL) was taken as the unit of measurement. Whole human specimens ranged from 28 mm CRL to 76 mm CRL; albino rat specimens ranged from 16.6 mm to 19.2 mm CRL; serially sectioned human specimens ranged from 34 mm to 55 mm CRL.

A procedure adapted by Watson (1977) for in toto double staining with alcian blue and alizarin red S for cartilage and bone was used on the fresh human and rat specimen whereas the serial sections of human specimens were stained with H & E. Two rat embryos were sectioned following staining with the above method (Watson 1977) and one human specimen which did not respond to the Watson Technique (1977) was also serially sectioned.

Results of the study support the view that although there is no exact time for the appearance of neural arch ossification centres in

the human, the sequence of ossification does appear to occur in two main regions, from three groups (as outlined above) and therefore not in a cranio-caudal direction.

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CHAPTER I

INTRODUCTION

The initial purpose of the present project was to investigate the early development of a lumbar vertebra in the human fetus. As the study developed, it became apparent that there was an inconsistency in the literature regarding the developmental pattern of ossification in the human vertebral neural arch centres, therefore this paper will concentrate solely on the latter topic.

Much research has been directed to the development of the human skeleton through stain techniques, radiographic and anatomical examination and histological sectioning. Due to legal and ethical considerations, human material has generally been unavailable in sufficient quantity to enable large comprehensive surveys to be conducted. Most of the research has been done on small numbers of specimens or laboratory animals resulting in inaccuracies and inconsistencies in the literature and the call for more data.

Current textbooks describe osteogenesis of the human vertebral neural arches as occurring in a cranio-caudal direction similar to the formation of the somites. In a recent large radiographic study of 195 human specimens, Bagnall (1977) concludes that neural arch ossification occurs from 3 main groups of centres. He suggests that the first group appears in the lower cervical/upper thoracic region quickly followed by a second group in the upper cervical region. The remaining cervical neural arch centres then ossify and osteogenesis spreads caudally into the thoracic region. Shortly thereafter a third group

of centres appears in the lower thoracic/upper lumbar region and ossification spreads cranially and caudally but more rapidly in the former.

In contrast to the findings for the vertebral neural arch ossification centres he supported the textbook descriptions (Williams & Warwick 1973) (Barson 1974 and others) of centra ossification centres appearing first in the lower thoracic/upper lumbar regions.

The object of a further series of experiments is to determine if the latter study can be substantiated through use of an alizarin red S and alcian blue stain technique, as modified by Watson (1977).

FETAL AGE

There appears to be no accurate method of estimating human fetal age unless exact data of coitus and menstrual history is available (Hamilton & Mossman 1972). With this in mind, it is not the intention of this study to investigate thoroughly the problem of prenatal age or to make a comparison with the sequence of ossification. However, an account of the various measurement techniques employed, seems pertinent.

It has been traditional to calculate fetal age from the date of onset of the last menstrual period (LMP) (Mall 1918, Birbeck et al. 1975). However, due to unreliable recall of the mother (Mall 1906, Arey 1939, Beazley & Underhill 1971) the time and date of conception cannot be accurately timetabled. Conception has occurred when the ovum is fertilized and time of ovulation has been found to take place mid-cycle, with a range from 8-19 days (Brewer & Jones 1947).

Streeter (1920, 1942, 1945, 1948, 1951) attempted to determine the age of an embryo from its length, but noted considerable variation.

Streeter has developed 23 Horizons in Human Embryos which depended on the degree of maturity established in the embryo. These "horizons" have been used extensively in embryonic study, but are limited in their use in bony development because they terminate at what he suggested as the end of the embryonic period, namely onset of ossification in the shaft of the humerus.

Other methods of measurement available to the worker include:

a. CRL (crown-rump length) - sitting height; vertex of the skull to the breech (midpoint between the apices of the buttocks)

b. CHEL (crown-heel extended length) - standing height; vertex of the skull to the base of the heel

c. NRL (neck-rump length) - greatest length taken with exaggerated forward spinal curves

d. Mean foot lengths

e. Birth weight (Pitkin 1969)

f. Ultrasound (Robinson 1973) - in utero

g. Limb lengths (Scammon & Calkins 1929)

h. X-ray

Formalin-fixed embryos may be subject to discrepancies and Streeter (1920) added 5% as compensation for fresh weight. Scammon & Calkins (1929) on the other hand suggest that preservation in formalin does not significantly affect specific measurements such as CRL.

These measurements are subject to genetic, nutritional and general environmental factors and no one measurement should be considered final (Hamilton & Mossman 1972).

DEVELOPMENT OF A TYPICAL HUMAN VERTEBRA

Introductory Outline - See Figures Nos. 1 and 2.

The vertebrae are mesodermal derivatives of the ventro-medial part of paired somites, the sclerotome. The cells of the sclerotome migrate ventrally and dorsally to surround the neural tube and fuse with its corresponding sclerotome about the notochord.

The largest accumulation of migrating cells lie ventral to the neural tube and constitute the primordia of the vertebral bodies and intervertebral discs. Each segmental sclerotomic mass becomes divided by fissures into equal cranial and caudal halves. A fusion, or rearrangement occurs in which caudal and cranial halves of successive vertebrae unite.

Cellular prolongations pass dorsally, to surround the spinal cord as the neural arch primordia, and laterally as the primordia of the costal processes.

With this rearrangement of segmentation, the vertebral bodies come to lie between the myotomes. The neural arches maintain a position nearer the superior surface of the vertebral body, where they attach. About the 6th week, six centers of chondrification appear in this mesodermal condensation. Two centres appear lateral to the notochord and quickly coalesce around it to form the chondrous centrum. Two centres appear lateral to the neural tube and spread dorsally to establish the neural arch and spinous process. Two centres appear at the junction of the neural arch and centrum and spread laterally to form the transverse processes. These cartilage masses later join one another and present a cartilage model of the definitive vertebra.

Primary ossification commences during the 8th week of development from three centres; one centre in the region of the vertebral body and one in each half of the neural arch. These centres spread until the only cartilage that remains is situated dorsally, in the midline, and at the junction of the neural arches and centrum.

Detailed Considerations

Development of the vertebral column, from intraembryonic mesoderm, is said to occur in three stages: 1. blastemal (membranous, prechondral, prochondral) 2. chondrogenous (cartilaginous) 3. osseogenous (ossification).

Blastemal

Provertebral mesoblast surrounds the notochord (chorda dorsalis) in a continuous nonsegmented type of investment. The notochord acts as a framework about which the primordial blastemal vertebral column is formed.

Upon elaboration of the notochord and neural tube, the mesoderm to the side of these structures, thickens to form two longitudinal columns, the paraxial mesoderm. This layer subsequently encloses the notochord and sends prolongations dorsally around the neural canal and ventro-laterally as the primordium of the neural arches and costal protrusions. Mesoderm extending lateral to the paraxial mesoderm proliferates as two other columns so that by the 19th day, three distinct zones of mesoderm are evident on each side of the notochord; the medial paraxial column, an intermediate column and a lateral mesodermal plate. The lateral plate mesoderm forms the layers (somatic and

splanchnic mesoderm) which enclose the coelomic cavities (evolving into pericardial, pleural and peritoneal cavities) while the intermediate column gives rise to the urogenital structures. The paraxial mesoderm gives rise to the somites which begin forming at about the beginning of the fourth week or nineteenth day (O'Rahilly 1972, Streeter 1945). Appearance of the mesodermal somites marks the beginning of metamerism, or arrangement of the body in segments. The first pair of somites appear in the occipital region just caudal to the rostral end of the notochord and proceed in a cranio-caudal direction until eventually 42 to 44 pairs of somites have formed. This happens rapidly over a period of about 10 days (Streeter 1942, 1945, 1948) and results in about 4 occipital, 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 8-10 coccygeal somites. The rostral end of the paraxial mesoderm does not undergo segmentation but forms the cranial bones, certain muscles of the head and connective tissue.

The somites appear as a series of cuboidal or square elevations along the dorsolateral surface of the embryo. In section, a small lumen, the myocoele, is said to occur in the center of each wedge-shaped somite and this increases in size until the somite appears as a hollow vesicle with thick walls. The wall differentiates into a dorsolateral part, the dermo-myotome and a ventromedial part, the sclerotome. Each sclerotome is separated by an intersegmental artery. The dermo-myotome contributes to the integument and voluntary muscle of the trunk, neck and part of the head; the sclerotome takes part in the development of the axial skeleton (base of skull, vertebra, sternum, ribs and costal cartilages). Spinal nerves are segmental in position as opposed to the arteries.

Each of the sclerotomes (ventromedial cell mass) migrates toward the midline, surrounds the notochord and fuses with its contemporary on the opposite side as a mesenchymatous tube, or anlage of the vertebral centrum. A longitudinal cylinder of circularly arranged cells around the notochord from end to end forms the perichordal sheath.

The problem of rearrangement in the vertebral column has been a subject inciting interest since the mid-19th century. Early development of the vertebral column is generally considered as a two-phase process; a temporary first generation rudimentary vertebra is replaced by a second generation definitive vertebrae with newly-situated segmental boundaries. According to Warwick and Williams (1973) the early sclerotome becomes separated into equal cranial and caudal halves by a transverse division, the sclerotomic fissure (also referred to as "intervertebral fissure"). The loosely packed caudal portion of each sclerotome unites with the loosely packed cranial portion of the adjoining sclerotome to form a single definitive vertebra. The point of fusion represents the vertebral centrum. The mesoderm on either side of the sclerotomic fissure condenses, and with that portion of the enclosed notochord, becomes the intervertebral disc.

The intersegmental arteries, lateral to the interval between the somites, eventually straddle the midsection of each vertebral body whereas the spinal nerves and myotomes become related to two vertebrae. Remak (1855) called the reconstruction "rearrangement" and the appearance of "intervertebral fissures" described by von Ebner (1889) marks the beginning of this rearrangement. Other researchers supporting the principle of rearrangement include Bardeen (1905), Sensenig (1949),

Prader (1947), Peacock (1951) as well as most textbooks (Warwick & Williams 1973, Hamilton and Mossman 1972 and others).

Remak (1855) describes the neural arches as developing laterally from the vertebral bodies, initially attached to the caudal half of the primitive vertebral bodies and then through appearance of "fissure-like gaps" and subsequent rearrangement, becoming appendages of the cranial aspect of the definitive vertebra. von Ebner (1889) finds "intervertebral fissures" running medially from the dermomyotomes and cites these as proof of rearrangement as "no element of form is to be found and they correspond to the boundaries of the later vertebrae". He also presents a functional argument in a second study wherein he alleges the rearrangement as necessary in order that the myotomes be situated between neighboring definitive vertebrae.

Prader (1947) using sagittal sections of five human embryo accords with Bardeen (1905) suggesting that indeed a rearrangement takes place however "not a mere regrouping of rigid sclerotome halves but a dynamic, flowing reorganization". With reconstructed schematic frontal section diagrams he develops a "shift theory"; that is, there is a gradual shift of zones of concentrated tissue mass, cranially, with a loosening of the tissue caudally. This dense zone is situated on both sides of the intervertebral fissure which has in the meantime disappeared and finally, alternates with the muscle segments. The disc, therefore, develops out of adjacent parts of the cranial and caudal sclerotome halves.

Sensenig (1949) in a large study of 88 embryos and fetuses, interprets the rearrangement in a similar way to Remak (1855) and von Ebner (1889) however concludes that the definitive vertebral bodies are

made up of two-thirds caudal and one-third cranial halves of the sclerotomes. According to the theory of rearrangement, the first four somites coalesce, the first disappearing completely, to form the occipital bone. The fifth and sixth somites coalesce with the upper half of the seventh to form the atlas and axis. All succeeding somites give rise to the remaining vertebrae and their intervertebral discs.

The occurrence of rearrangement, however, has been questioned by some authors (Williams 1908, Baur 1969, and Verbout 1972). Baur (1969) and Verbout (1972) suggest that the vertebrae develop from the blastema and take up their definitive positions from the outset without any evidence of rearrangement. Baur (1969) also suggests that von Ebner's (1889) "intervertebral fissures" in the ring snake are artefacts and could not be confirmed histologically in his own study using ring snake embryos. Baur (1969) likewise could not confirm Prader's (1947) description in which he suggests that the zones of density shift cranially within the tissues.

Williams (1908) does not concur with the idea of resegmentation and states that following the migration of cells about the notochord, there is no period of condensation and formation of provertebrae. He believes that the segmentation of the vertebrae is definitive from the beginning and that the early demarcation of vertebrae occurs as a result of differential growth of mesenchymal cells.

The neural arches exist as segmental condensations between the spinal ganglia and the separation into ribs and vertebral arches can be seen shortly before the cartilaginous stage (Baur 1969). Thus the individual vertebrae are derived from three cell mass extension of the

original sclerotome; a first group of cells migrating ventro-medially to form the vertebral centrum; a second group migrating dorsally to form the neural arches; a third group migrating ventrolaterally to form the costal processes and in the thoracic region, the ribs. All vertebrae are capable of bearing ribs, however in mammals, although it is not uncommon to note a lower cervical or upper lumbar rib, only the thoracic vertebrae normally display ribs.

The addition of cytoplasm and rapid differentiation of cells forming the anlage of the centrum, is believed to be a result of more nutrition due to closer proximity to the intersegmental arteries. With the rapid addition of cytoplasm, a difference in pressure is exerted on the notochord. Cells of this structure are extruded into the intervertebral regions where the pressure is less. Mucoid degeneration of notochordal cells appears and in older embryos, only a mucoid streak remains at the site of the vertebral bodies. Occasionally a few remnants of notochordal cells are found in the substance of the adult vertebral body.

Chondrogenous

It is generally agreed (Dodds 1946, Williams & Warwick 1973, Rothman & Simeone 1975) that in the sixth week of embryonic life, six centres of chondrification appear in the mesenchyme of the human membranous vertebral column. Appearing are two centres in the body lateral to the notochord, one centre in each neural arch lateral to the neural tube and one centre at the union of the arch and centrum. These rapidly coalesce to complete: the chondrous centrum, neural arch and spinous process, transverse and articular processes.

The vertebra is no longer a condensed mesenchymal or membranous vertebra. It basically represents at this point, a cartilaginous model of the future ossified vertebra. The cartilaginous laminae do not meet until the fourth month of intra-uterine life and are joined until then by a closing membrane which defines the spinal canal.

Bardeen (1905) describes the transverse process as a short projection during the chondrogenous stage, lying some distance from its corresponding rib. The rib grows rapidly and at the same time the transverse process grows ventrolaterally to meet it, forming the future costo-transverse joint. By contrast, the articular processes develop slowly.

The part of the notochord in the centrum disappears so that, prior to ossification, only the perichordal sheath remains as the mucoid streak. According to Keyes and Compere (1932) the notochordal material is henceforth considered intervertebral as the nucleus pulposus. The surrounding fibrocartilage develops as the annulus fibrosus.

Recognizable as a separate entity subsequent to chondrification is a structure named the hypochordal bow which connects the vertebral ends of two costal processes ventral to the centrum. This persists as a special feature, the anterior arch of the first cervical vertebra (atlas) and disappears or becomes incorporated in the anterior part of the annulus fibrosus in succeeding vertebrae. The centrum of the atlas joins the second cervical vertebra (axis) and chondrifies to form the bulk of the dens.

Osseogenous

As with other skeletal bones, this involves both primary and

secondary ossification centres and is endochondral in manner. It is generally accepted that the typical vertebra develops from three primary centres, one for the body and one for each arch, unless the costal processes as an additional two centres are included.

Ossification begins in about the ninth week of intra-uterine life (Davies 1973 suggests seventh week) by the invasion of vessels into the ossification centre. Typically, a vertebra is supplied by segmental arteries arising near it from the aorta or from adjacent arteries in areas beyond the extent of the aorta. A segmental artery courses dorsally along the lateral surface of the body of a vertebra giving off branches to it and dividing near the inter-vertebral foramen into 1. a lateral branch, a continuation of the segmental artery and 2. a spinal branch. The spinal branch, of concern here, enters an inter-vertebral foramen and divides into three branches: a dorsal, intermediate and ventral. The dorsal branch anastamosing with similar branches above and below, supply the vertebral arches and structures within the neural canal with the intermediate branch. The ventral branch subsequently divides into ascending and descending branches which course towards the centres of two adjoining vertebrae. A branch from each side, between the posterior longitudinal ligament and posterior surface of vertebral bodies enters the middle of the vertebral body.

Veins make up a rich plexus, extending the length of the column both inside and outside the neural canal. There is free communication with the veins of the body wall and relatively few valves.

Onset of ossification is generally described as commencing in the neural arch centres, situated at the base of the transverse process and spreading out in a triradiate fashion; dorsally into the lamina,

ventrally into the pedicle and laterally into the transverse process. This is said to occur initially in the upper cervical region spreading rapidly in a cranio-caudal direction (Williams & Warwick 1973) similar to the development of somites. Bagnall (1977) however, reported a sequence of ossification as spreading from two basic regions; the cervical and lower thoracic/upper lumbar. Shortly after, ossification centres appear in the bodies of the vertebra of the lower thoracic/upper lumbar regions spreading in each direction however more rapidly in a caudal direction. The centrum may show a dorsal and ventral component for a short time, separated by a plate of cartilage. By the 16th week ossification is well under way. The two halves of the neural arch do not unite dorsally until the first year of postnatal life and remain separated from the centrum by cartilage until after birth (3-6 years).

Five secondary centres of ossification appear during puberty with the appearance of two epiphyseal centres in the body of each vertebra as well as others in the tips of the spinous and transverse processes. With the extension of the primary ossification centres, a typical vertebra at birth consists of three main ossified elements: a centrum and the halves of the neural arch. These are united by cartilage. The upper and lower surfaces of the centrum and the tips of the transverse and spinous processes remain cartilaginous until puberty. Ossification is completed at about the 25th year of life. Cartilage plates on each end of the vertebral bodies are residues of the primitive, entirely cartilaginous vertebral body.

Previously, reference was made to that portion of the notochord which was gradually compressed during chondrification. Further

compression occurs during ossification of the definitive vertebral body and cells of the notochord are now confined normally to the central portion of the intervertebral disc although eccentrically placed in a ventral position during fetal life. Luschka (1858) maintained notochordal cells could be found at all stages of life in the middle of the disc. Peacock (1951) states they are occasionally found near the intervertebral region.

Contributions dealing particularly with the development of the intervertebral disc are scant prior to Prader (1947) and make only references to prenatal stages (Keyes & Compere 1932, Tondury 1947). Prader (1947) describes the intervertebral disc as a structure composed of three zones: outer, middle and inner, rooted in the cartilaginous disc. He maintains that in the early embryonic stage, the outer lamellar zone is entirely fibrous with an inner zone temporarily built up of hyaline cartilage, distinguishable from the hyaline cartilage of the vertebral body. In a later embryonic phase, the middle zone of primitive fibrous cartilage forms which develops from the outer but mainly inner zones and is transformed into true fibrous cartilage at birth or later. He believes the nucleus pulposus evolves from a liquefaction of the central part of the hyaline cartilage, amalgamated with the mucogelatinous mass of chordal origin.

Bardeen (1905) refers to a 'primitive disc' formed by a joining of condensed portions of the sclerotome halves however main discussion centres on the development of the vertebrae.

Peacock (1951) conducted a reasonably large study of 38 human embryos and fetuses ranging from 3 mm CRL to full term, from which he substantiates the view of Prader (1947), that is, the existence of

three zones: outer, middle and inner.

The role played by the notochord in the formation of the nucleus pulposus seems to be a matter of controversy. Conclusions drawn vary from complete absence to exclusive participation of notochord cell material in the formation of the nucleus pulposus. It is not within the text of this paper to investigate the subject. The nucleus pulposus is avascular during fetal and adult life, being nourished by diffusion. The vertebrae are freely vascularized and the upper and lower faces, covered with cartilaginous plate, completely separate the vascularized areas of the disc (annulus fibrosus) from the vessels of the vertebral bodies (Rickenbacher 1969).

SUITABILITY OF STAIN TECHNIQUE AS A METHOD OF STUDYING FETAL CHONDROGENESIS AND OSTEOGENESIS

Schultze (1897) developed the first method of locating the appearance of bone with a good degree of certainty, by rendering embryos transparent. Mall (1906) states that "little is to be gained in reviewing the literature upon this subject for such specimens, since the results are not satisfactory when compared with a good series of Schultze specimens". Since then, stain techniques have been continuously improved.

Meyer & O'Rahilly (1958) compared three different methods for determining the onset of ossification. Assuming that the onset of ossification was similar on both sides of the body, four human fetuses were bisected: 1. one half of each fetus was stained with alizarin red following clearing; 2. the other half of each fetus was impregnated with silver, radiographed; and 3. finally serially sectioned for

histological study. Histological sections produced the best results. Staining with alizarin red appeared to provide slightly better results than the radiographic method. They further concluded that a centre of ossification was present only with the advent of the onset of endochondral ossification. The laying down of osseous deposit as a periosteal collar is considered intramembranous and until invasion of the cartilage by the periosteal bud takes place, a centre of ossification cannot be considered as having commenced. The laying down of a periosteal collar is an indication of an impending site of endochondral ossification as it forms at the same time or slightly before (Gardner 1971). Bagnall (1976) suggests that from a study of his radiographs it was evident that periosteal and endochondral ossification develop together. Gardner (1971) states that ossification is detected slightly earlier in stained and sectioned material than in clearing of whole specimens although the latter method provides for beautiful specimens and rapid study.

By clearing in KOH and staining with alizarin red simultaneously, overmaceration and overstaining of human embryos and fetuses can be prevented (Noback 1951).

Although alizarin red is claimed to be nonspecific to calcium, it concentrates more heavily in areas of new osseous deposition and stains adult bone poorly except around a growing edge (Cameron 1930). Success of the method depends on obtaining a proper degree of clearing before staining; inadequate clearly results in general staining of all tissues (Dawson 1926). Alcian blue tends to remain in the tissues of formalin fixed and formalin fixed alcohol stored specimens, especially large vertebrates. Dingerkus and Uhler (1977) successfully employed a

technique for clearing the tissues of alcian blue in small vertebrates up to 30 mm CRL using trypsin prior to staining and alizarin red stain. Watson (1977) developed a modified technique using trypsin and was successful in demonstrating cartilage and bone in specimens up to 130 mm in length.

Histological methods applied to the rat are essentially the same as those used for the tissues of other mammals (Addison 1925).

PATTERN OF OSSIFICATION

All bones begin as mesenchymal condensations whether classified as membranous or endochondral. "Membranous bones" develop in a plexus-like manner where there is a preponderance of fibrous tissue. Endochondral or "cartilage bones" pass through an intervening stage of chondrification which produces a cartilage model before periosteal and endochondral ossification occurs.

The process of ossification is fundamentally the same wherever it occurs with basic underlying factors (Streeter 1949, Gardner 1971) although patterns of ossification may differ somewhat and onset is extremely variable. Mineralization of cartilage is only one of the stages in the process of osteogenesis and essentially involves destruction and removal of the cartilage precursor.

Bone is a specialized connective tissue consisting of a mineralized organic matrix in which living cells are scattered. This matrix is made up of collagen fibers within a ground substance containing protein mucopolysaccharide complexes. Calcium, phosphate and carbonate make up the mineral of bone; osteoblasts, osteoclasts and osteocytes represent the main cells of bone. The osteoblast is the cell

which is responsible for making bone by the synthesis of the collagen and ground substance.

Three separate, however co-ordinated processes occur in endochondral bone formation located at the periosteal collar, internal growth centre and epiphyses. Osteoblasts, differentiated from precursor cells on the inner layer of the perichondrium, produce a circumferential boney collar. The site of bone formation can be identified by the presence of both matrix synthesis and mineralization apparent, for one, by the uptake of special stains such as toluidine blue. At surfaces where bone formation is occurring is a layer of as yet unmineralized matrix, adjacent to the osteoblasts - the osteoid seam.

At the growth centre, the chondrocytes in various stages of development form zones of proliferation, hypertrophy and provisional calcification, arranged in columns perpendicular to the epiphyseal plates. Between the columns of cells, calcification occurs. That is, there is a movement of ions through preosseous matrix, a taking up of calcium by the cells and subsequent extrusion from the cell of calcifying globules and matrix vesicles. Eventually, mineralization is achieved by the deposition of a crystalline form of mainly hydroxyapatite, as longitudinal bars of calcified cartilage. The hypertrophic cells degenerate and capillary invasion of the spaces bring primitive mesenchymatous cells. These cells differentiate into chondroclasts, which partially resorb the bars of calcified cartilage and osteoblasts which deposit woven bone onto their surfaces.

Successive waves of remodelling involving resorption and replacement occur. By birth, osseous replacement of the vertebral cartilaginous model is basically completed. Remodelling by the osteoclasts on

the endosteal surface of the vertebral walls produce vertical trabeculae and extend existing horizontal trabeculae. Lateral expansion occurs as a result of intramembranous periosteal, appositional growth.

Cartilaginous plates lie at the caudal and cranial ends of each vertebra and between the neural arches and body, representing the equivalent of the epiphyseal growth plates of long bones.

The vertebrae are considered as irregular bones having been preformed in cartilage and covered with perichondrium usually before the end of the embryonic period. The cartilage model closely approximates the shape of the bone which will eventually replace it. Intrinsic factors responsible for the differentiation appear to be virtually unknown although pre-determined, depending upon their potencies and environment. The pattern of ossification in irregular bones differs somewhat from tubular bones. The vertebral bodies undergo endochondral ossification prior to periosteal bone formation, whereas in the vertebral neural arches they occur simultaneously (Gardner 1971).

Streeter (1949) outlined the morphological transformation of cartilage for the human humerus as occurring through five arbitrary cell phases. These were stainable under favorable conditions and recognizable before the cartilage acquired independent vascular supply. Fluid requirements were considered as being obtained by means of diffusion.

Phase I

Cartilage cells without specific arrangement and undergoing proliferation begin to acquire increased intercellular substance and are the "youngest" most peripherally situated cells.

Phase II

Cells are in active division, elongated and show traces of vacuolization. In the humerus, these cells were noted to arrange themselves in characteristic tiers, transverse to the long axis of the bone. In the vertebrae these centres constitute small groups of cell nests; the oldest cartilage cells are in the central part of the ossification centre, surrounded by zones of successively younger cartilage cells.

Phase III

Further hypertrophy and vacuolization of cartilage cells with a marked increase in inter-cellular substance.

Phase IV

Cartilage cells reach maximum size with extreme vacuolization and only a thin border marks the contour of the cell. The intercellular material is more opaque and resembles a honeycomb.

Phase V

Advanced disintegration and liquefaction of cells appears, some areas of the honeycomb being left with vacated compartments.

These changes in the cartilage are preliminary to the invasion of blood vessels and ossification. When onset of ossification occurs, the centres of ossification for the neural arches and ribs correspond to the original sites of chondrification in the blastema. However, the centres for ossification of the bodies show little resemblance to the original bilateral situation (Bardeen 1905).

Although a typical vertebra develops from three ossification centres,

the cervical vertebrae, 1 (atlas), 2 (axis) and 7 are exceptions:

1. Atlas - has three centres of ossification. However, onset occurs in each lateral mass prior to birth and in the anterior arch after birth.
2. Axis - ossification commences from one centre in each vertebral arch and the centrum whereas the dens, actually the centrum of the atlas, is ossified from two laterally placed centres. These are primary ossification sites appearing during intra-uterine life, followed by two secondary sites appearing after birth.
3. Cervical 7 - ossification sites for the costal processes are usually separate and may grow ventro-laterally as cervical ribs if they remain thus. Otherwise ossification centres appear in the neural arch and centrum as with the typical vertebrae. Centres for cervical 4, 5 and 6 costal processes have also been found (W. and W. 1973) however these are less common.

Schinz and Tondury (1942) conducted an exhaustive anatomical and radiographic study in which they demonstrated the relative position of early ossification centres in individual vertebrae of the human fetus. A series of excellent photographs were produced. There is some dispute in the literature (Cohen et al 1956) regarding the appearance of the early ossification centres of the vertebral body and three theories have evolved: 1. a single centre for each segment; 2. paired lateral centres; 3. dorsal and ventral centres. Nevertheless, the sequence of ossification of the vertebral bodies commences in the lower thoracic/upper lumbar regions and spreads both cranially and caudally from this area (N & R

1951, O'R & M 1956, W & W 1973).

Noback (1954) notes that although an extensive amount of literature appears to be available regarding the appearance times for ossification centres, the only original accurate data produced was that of Mall (1960), Augier (1931), Teissandier (1944) and Noback and Robertson (1951). The general concensus is that most of the primary ossification centres appear over a very short period of time before the end of the fourth month of intra-uterine life.

The developmental pattern of the neural arch ossification centres has evoked some disagreement. Most textbooks report that neural arch ossification centres appear in the upper cervical region and spread in a caudal direction from this region (W. & W. 1973, Davis and Dobbing 1974). Prior to 1944, with the exception of an observation made by Mall (1906) data were derived from specimens which had either no neural arch ossification centres present or at least nine cranial centres. The assumption was made that since ossification spread in a caudal direction from thoracic vertebra 2, this had been the case in the cervical region as well. Noback (1944) compared a specimen he encountered with the data of Mall (1906) and suggested that the sequence of ossification was not in a cranio-caudal direction and that possibly ossification commenced in first thoracic vertebra. At a later date, Noback and Robertson (1951) had encountered an additional five specimens in which there were less than nine neural arch ossification centres present and came to the same conclusion, although stating that more data were necessary. Teissandier (1944) conducted a study of three fetuses, concluding that cervical arches 2 and 7, thoracic arches 1 and 12 and lumbar arch 1 are the first arches to ossify. Bagnall (1976) in a larger study of 24 fetuses, in

which not all the ossification centres for the neural arches proximal to lumbar 2 had appeared, concluded, as Teissandier (1944) that the sequence of neural arch ossification spreads from the two basic regions, namely, the cervical and lower thoracic/upper lumbar regions.

Furthermore, in support of Noback and Robertson (1951) Bagnall concluded (1977) that no regular sequence for the appearance of ossification centres in the nine most cephalic neural arches is evident and determines that this includes all the thoracic and upper lumbar regions as well. He suggests that three groups of centres appear: a first group in the lower cervical/upper thoracic region, rapidly followed by a second group in the upper cervical region, then a third group in the lower thoracic/upper lumbar region.

In accordance with most textbooks (W. & W. 1973, Barson 1974) Bagnall (1976) substantiates the view that ossification centres of the vertebral centra first appear in the lower thoracic/upper lumbar region. No conclusion was arrived at as to whether or not the neural arch ossification centres appear before the vertebral centra ossification centres as reported by Barson (1974).

The occurrence of assymetry has been referred to by Pryor (1906) in which he records that ossification within the body is bilaterally symmetrical regardless of the normal variations. Noback (1944) came to a similar conclusion in which he accepted the fact that occasionally assymetry would occur but basically the appearance of bilateral centres is essentially symmetrical. Later, Noback and Robertson (1956) observed that a normal time exists for the appearance of an ossification centre and such an occurrence is not observed after that time. Christie (1949) suggested that any assymetry in the development of any particular centre

was insignificant and O'Rahilly and Meyer (1956) decided that an ossification centre would be considered present whether it be unilaterally or bilaterally situated.

Since human fetal material is so difficult to obtain, many researchers have turned to laboratory animals, for example, the rat (Strong 1925). Investigations done by Strong (1925) using alizarin red S stain, determined that ossification centres appeared in the arches of the cervical vertebrae and the first two lumbar vertebrae at 17 days + 8-1/4 hours. Ossification was more advanced in the cervical vertebrae, particularly the atlas, in its lateral portion. He further describes a second centre appearing "somewhat dorsal to this in all of the cervical vertebrae except the first two and in the first thoracic vertebrae of some specimens". Ossification centres then occur in successively more caudal vertebrae and relative to man, much later in embryonic life. First ossification occurs in the rat, less than one week before birth and progresses more rapidly than in man, once started.

CHAPTER II

MATERIALS AND METHOD

Material made available for this study consisted of ten unsexed human embryos and fetuses, eighteen albino rat embryos and 108 human specimens, serially sectioned, from the Dr. R.F. Shaner collection.* There were two fresh frozen human fetuses and all of the remaining human specimens had been collected and stored for a lengthy period of time in a 10% formalin solution. Embryonic age was calculated on the basis of crown-rump length (CRL), the total number ranging from 27 mm to 75 mm CRL. Since human fetal material is so difficult to acquire, 18 albino rat fetuses were used, ranging from 16.6 mm to 19.2 mm CRL, as a means for comparison and supplemental study. The fresh frozen and fresh human and rat specimens were subsequently fixed in an acetic acid-alcohol solution.

The present work was undertaken with the intention of determining the sequence of ossification of the human vertebral neural arch centres using an in toto alcian blue and alizarin red S combination stain technique, put forward by Watson (1977) for demonstration of the cartilaginous and bony skeletons in mammals. Prior to commencing the procedure on human material, it was tested successfully on two newborn albino rats. Furthermore, the process was subsequently reversed to determine if deeper staining of cartilage and bone could be achieved. Results were satisfactory.

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*Henceforth referred to as the RFS collection, Dept of Anatomy, University of Alberta, Edmonton.

The technique consisted of four basic steps:

1. fixing and staining
2. dehydration
3. clearing
4. final clearing, staining and storage

HUMAN SPECIMENS

A. FORMALIN FIXED - See Table I.

1. Staining

Following washing in distilled water for at least one day, the embryos were transferred directly into a solution consisting of:

Ethyl alcohol 95%	80 ml
Glacial Acetic Acid	20 ml
Alcian Blue 8GN	15 mg

Small embryos up to 40 mm CRL remained in this solution for about 24 hours; medium and large-sized embryos up to 48 hours

Of the eight specimens, two (A & K) were left intact throughout the entire procedure, two were eviscerated before staining (I & J) and four were given vertical abdominal slits with skin perforations about the spine (D, F, G and H). The latter four embryos were subsequently eviscerated either after the alcian blue staining or during the final clearing phase, to facilitate visibility of the vertebral column.

2. Dehydration

Over a period of one week all specimens were placed in 95% ethyl alcohol with three changes.

3. Clearing

According to a technique designed by Dingerkus and Uhler (1977) specimens were placed in an enzyme solution of:

Saturated aqueous sodium borate	30 ml
Distilled water	70 ml
Trypsin (approximately 1/4 tsp.)	1 gm

The solution was changed every 4-5 days or whenever it became discoloured. The specimens remained in this solution until all the blue had been removed from the soft tissues and the skeleton was clearly visible.

4. Final Clearing, Staining and Storage

Prior to staining, specimens were placed in a solution of potassium hydroxide for one day, the strength graded according to crown-rump length:

A	.25%
D, F and G	.5%
H, I, J and K	.5%

Bone staining was then incorporated into the potassium hydroxide clearing step using the routine alizarin red S method (Dawson 1926) and the tissues were transferred to a solution of 0.1% aqueous alizarin red S in 1% potassium hydroxide. After the specimens were allowed to remain in the stain for a period of time averaging 40 minutes, the

embryos and fetuses were passed through a graded series from potassium hydroxide to pure glycerine, until the soft tissues became completely transparent. A few drops of hydrogen peroxide (3%) were added during the initial stage of the series to bleach out dark tissue pigments.

Specimen F demonstrated minimal staining in the long bones of the appendicular skeleton thus the process was reversed and repeated three times, using fresh solutions each time. The alcian blue stain for cartilage had almost completely disappeared by the end of these three processes. In the third and final process, seventy-two hours in alizarin red S solution failed to produce a red stain in any ossification centre. Thus, it was decided to pass the embryo through an alcohol-chloroform series in preparation for embedding and sectioning in anticipation of finding bone cells in the neural arches histologically. See Section C - Histological Serial Sections.

B. FRESH FROZEN - See Table I.

1. Fixing and Staining

Frozen specimens B and C were placed in a 10% formalin solution and left for 24 hours with one change. These embryos showed signs of "freezer burn".

Following washing in distilled water for two days with three changes, they were placed directly into a freshly prepared solution of:

Ethyl Alcohol 95%	80 ml
Glacial Acetic Acid	20 ml
Alcian Blue 8GN	15 mg

and kept for 24 hours.

Fresh and fresh-frozen embryos up to 40 mm CRL are stained for 20 hours and larger specimens can be stained for up to 48 hours.

2. Dehydration

As with the long term formalin fixed specimens, B and C were dehydrated in 95% ethyl alcohol for one week with three changes.

3. Clearing

With Alcian Blue fixed embryos, very weak aqueous solutions of potassium hydroxide are adequate as they are much more delicate than the formalin fixed embryos. The solution is changed when it becomes discoloured. Small and delicate specimens may be cleared with more control in a very weak potassium hydroxide solution to which has been added 5-10% glycerine (Watson 1977).

With this in mind, the embryos were placed into a .25% potassium hydroxide solution which was changed four times over a period of 21 days. After ten days of clearing, all skeletal parts were removed, leaving only the vertebral column, the basi occiput and ribs. Fleshing out of excess embryonic musculature was also done.

4. Final Clearing, Staining and Storage

Following the initial clearing step, 10 mg of alizarin red red S stain was added to a .25% potassium hydroxide solution and specimens B and C remained in this solution for four hours. They were then cleared in a four-step graded series from .25% potassium hydroxide solution to pure glycerine over 17 days. A few drops of hydrogen peroxide (3%) were added to the clearing mixture, initially, although dark tissue pigments were not present.

C. HISTOLOGICAL SERIAL SECTIONS

Specimen F - See Table II

As mentioned earlier, specimen F failed to absorb alizarin red S bone stain. It was decided to pass the specimen through a standard chloroform to paraffin wax series and embed, stain and serial section it at 12μ in a coronal plane. Chloroform was chosen instead of xylene, as test specimens (Rat) processed in xylene had appeared hard and less easy to section.

Three stains were tested on specimen F using two sections for each:

H & E - Hematoxylin and Eosin

HPS - Hematoxylin Phloxine Safran

Koneff's - Aniline Blue Stain with Methyl Green

Koneff's stain for cartilage and bone was successful in demonstrating hyaline cartilage and connective tissue, although the nuclei in the cartilage remained colorless. In areas where the cartilage appeared to be undergoing terminal hypertrophic changes, very few

unstained nuclei were observed in the larger vacuolated cells and it was assumed that these had been washed away in the original or final staining process. The surrounding connective tissue appeared as a reticulum without evidence of nuclei.

Technique

Paraffin or celloidin embedding.

Solutions:

5% Iron Alum Solution

Iron alum	5.0 g
Distilled water	100.0 ml

Methyl Green Solution

Methyl green, 1% aqueous solution	2.0 ml
Glycerin	2.0 ml
Distilled water to make	50.0 ml

Aniline Blue Solution

Aniline blue, water soluble	0.1 g
Oxalic acid	2.0 g
Phosphomolybdic acid	15.0 g
Distilled water	300.0 ml

2% Phosphomolybdic Acid Solution

Phosphomolybdic acid	2.0 g
Distilled water	100.0 ml

Procedures:

1. Dewax and rehydrate sections to 70% alcohol.
2. Treat with iodine-sodium thiosulfate technic if mercury containing fixative was used.
3. Wash well in water.
4. Place in 5% iron alum solution for 5 minutes.
5. Rinse in distilled water for 1 minute.
6. Stain in Harris' hematoxylin for 10 minutes.
7. Wash in distilled water.
8. Stain in methyl green solution for 20 minutes.
9. Wash in distilled water.
10. Stain in aniline blue solution for 20 to 40 minutes. This solution both stains and differentiates the tissue. The extent and degree of staining is checked under the microscope after rinsing the slide in distilled water. Staining should be carried on until the recently laid down bone is blue and the old bone is red.
11. Wash in distilled water.
12. Wash in 2 changes of absolute alcohol to which two drops of 2% phosphomolybdic acid solution has been added for each 50 cc of alcohol.
13. Dehydrate clear and mount.

Results:

Hyaline cartilage and immature bone	blue
Bone	bright red or reddish brown
Matrix of epiphyseal disc and remnants of matrix	green
Proliferating cartilage	blue

RFS COLLECTION

A total of 108 sets of human embryos and fetuses, serial sectioned and stained with H and E were examined. These specimens had a range from 1.5 mm to 170 mm CRL. Out of this number were selected only those specimens which illustrated pre-ossification and early ossification periods in the spine. Five sets which were clear in detail were isolated, ranging in CRL from 34 mm to 55 mm. Many of the specimens were incomplete, in that torsos were without head and neck or vice versa. The thickness of the sections in some specimens also varied. However, this was not felt to have an effect on the results. All are labelled with an "H".

Graphs were produced (see Tables 3 to 7 indicating the number of sections over which each specific vertebral arch appeared to be in the process of mineralization or ossification. In these sections, margins of a growth centre were set as vacuolization of cartilage cells either appeared, or disappeared, as opposed to Specimen F.

The general shape of the curves on the graphs were felt to be indicative of the various stages of development of the vertebral neural arch pre-ossification centres and would be sufficient for comparison as to which centre had commenced mineralization first.

ALBINO RAT SPECIMENS

A. FRESH SPECIMENS

Two female white albino rats (W - 332 g; R - 357 g) were mated with males of the same stock and pregnancy was confirmed by vaginal smears. The presence of spermatazoa in the vaginal smear was considered to indicate day 1 of pregnancy. Pregnant animals were then housed together but separated from the males.

On days 17 + 7 hours to 17 + 22 hours, approximately 4 days before term, rat W was killed with chloroform and six fetuses ranging in CRL from 16.9 mm to 19.2 mm (and averaging 18.05 mm) were extracted by caesarian section. These were considered as Lot W and numbered 1 to 6.

On days 17 + 40 minutes to 17 + 13-1/2 hours rat R was killed with chloroform and twelve fetuses ranging in CRL from 16.6 mm to 19.2 mm (and averaging 17.92 mm) were extracted by caesarian section. These were considered as Lot R and numbered 7 to 18. Measurements were taken with anthropometric calipers from the vertex of the skull to the cranial end of the tail. The difference in spinal curvatures was not taken into consideration.

All fetuses were immediately placed in a solution consisting of:

Ethyl alcohol 95%	80 ml
Glacial acetic acid	20 ml
Alcian Blue	15 mg

Lot W and R remained in the fixative for 48 to 54 hours respectively, following which they were dehydrated over one week in three changes of 95% ethyl alcohol. From this stage on, the procedures were altered.

LOT W - Numbers 1 to 6

Fetus #1 (18.3 mm CRL) was eviscerated and placed for two hours in a solution made up of 10 mg alizarin red S in 0.5% potassium hydroxide. The tissues were then satisfactorily cleared in three changes over 11 days of:

Potassium hydroxide	0.25	g
Glycerine	20	ml
Distilled water	79	ml

Following the clearing stage the fetus was prepared for embedding in paraffin by transfer through an alcohol and chloroform series: see section B - Histological Serial Sections (Rat).

Fetuses #'s 2 to 6 (19.2 mm; 18.2 mm; 17.5 mm; 16.9 mm; 18.2 mm CRL, respectively) were eviscerated and cleared in a similar solution to fetus #1, then mounted in pure glycerine without being passed through the alizarin red S bone stain. This procedure was implemented to see if the earliest stages of ossification were visible regardless of the bone stain as Strong (1925) had noted in unstained material. The earliest stages of ossification were not clearly visible. Therefore at a later date, fetuses #'s 2, 4, 5 and 6 were also passed through the routine alizarin red S bone stain procedure, following reversal of the alcohol to pure glycerine process.

Fetus #3 (CRL 18.2 mm) was not stained for bone but passed through a xylene to paraffin wax series and subsequently embedded and serially sectioned. See below - Section B - Histological Serial Sections.

LOT R - Numbers 7 to 18

All fetuses were stained for bone in a solution of 10 mg alizarin red S and 0.25% potassium hydroxide and remained in this mixture for four hours. Final clearing of tissues was accomplished in nine days using the graded weak potassium hydroxide solution (0.25%) to pure glycerine series.

The spines of all fetuses were removed and isolated from the surrounding bony framework as the head, thorax and limbs were found to obstruct detailed observation of the neural arches.

B. HISTOLOGICAL SERIAL SECTIONS - See Tables VIII and IX.

Sagittal serial sections at 14 and 20 m respectively, were made of fetuses #'s 1 and 3, following the initial clearing stage, by transferring each through the succeeding process with the exception of fetus #2 in which xylene was substituted for chloroform. Chloroform rendered fetus #1 easier to section as the tissue was less rigid.

Procedure:

50% alcohol	- 2 changes over 1 day
70% alcohol	- 2 changes over 1 day
95% alcohol	- 2 changes over 2 days
Absolute alcohol	- 2 changes over 2 days
Chloroform	- 1 day
Half chloroform + half paraffin was	- 1 day
Paraffin @ 56°C	- 4 changes over 2 days

Results were satisfactory in showing differentiation of cartilage

cells although dark tissue pigments were scattered throughout each section in fetus #3.

PHOTOGRAPHY

Slides were taken with a Nikon FTN camera, using a 55 mm micro-nikkor lens and ring reversal. Type R positive prints were subsequently made and dry-mounted.

Most slides were taken between f16 and f8, using either a flash or 40 watt bulb directed through a frosted glass table.

All specimens were immersed in pure glycerine, during the procedure.

CHAPTER III

RESULTS AND OBSERVATIONS

HUMAN SPECIMENS

A. FORMALIN FIXED AND FRESH FROZEN - See Table I and photographic plates.

1. Staining

Eight formalin fixed human specimens, lettered A, D, F, G, H, I, J and K ranging in crown-rump length from 27 mm to 76 mm were stained with alcian blue for demonstration of cartilage.

In the amount of time proposed, all specimens except F acquired a bright blue colour and when studied through a binocular dissection microscope with the light beam directed from below the glass table plate, the cartilaginous structures were seen to be a deep blue.

From the beginning, specimen F (53 mm CRL) presented problems in absorbing stain and required three changes of frf fresh alcian blue solution over three days. Even then, the cartilaginous skeleton was not as blue as desired. However, it was felt that the soft tissues would become irreversibly stained if left in yet another change of solution.

It was decided to pass specimen F as the other embryos and fetuses, through successive steps of the procedure, regardless.

Two fresh frozen embryos, B and C, ranging in CRL from 50 to 60 mm, were stained with Alcian Blue following immersion in a solution of formalin for 24 hours. Crown rump length could not be accurately determined as the material had been obtained by suction. Only the spinal columns with attached pelvis, lower limbs and partial thorax were essentially intact but divided into two pieces in the upper thoracic region. By comparison of costal andinnominate bone ossification centres with other intact embryos, a crown-rump measurement was estimated and given.

After 24 hours' staining, B and C exhibited (under the binocular dissection microscope) a deep blue staining of the cartilages when viewed. The soft tissues at this time appeared opaque and whitish-blue in colour. The blue colouration achieved in the cartilage was much more vivid than in the formalin fixed embryos and did not leach out or fade in subsequent stages of the procedure. The "freezer burn" mentioned earlier did not seem to affect these specimens adversely.

2. Dehydration

For both formalin fixed and fresh frozen specimens (A through K) the tissues became moderately rigid in three changes of 95% ethyl alcohol over one week.

3. Clearing

The amount of time required for clearing of formalin fixed specimens ranged from 6 days and 3 changes for specimens A, D and G to 20 days and 6 changes for specimens H and I. The

latter two were periodically skinned and fleshed out in the region of the spinal column. However, they continued to retain an evenly opaque appearance. Specimen F required nine days and three changes for satisfactory results, except that the Alcian Blue stain seemed to have faded or leached out when compared to other specimens.

Specimens B and C, cleared in very weak aqueous solutions of potassium hydroxide became fairly transparent. With the removal of excess skeletal tissue and limbs, the vertebral column was readily visible. Areas assumed to be ossification sites, gave the appearance of being much clearer, roughened and less stained although with a slight brown tinge in some cases.

4. Final Clearing, Staining and Storage

Formalin fixed embryos and fetuses cleared fairly rapidly even though weak solutions of potassium hydroxide were used. Most required in the neighborhood of 27 days. Specimens H and I retained some opacity. Ossification centres were visible through the tissues as excess soft tissue had been removed. These two specimens required 35 days in the clearing solution.

The viscera in the intact specimens (A and K) failed to clear adequately. Neural arch ossification centres were readily visible, however, vertebral centra ossification centres were moderately obstructed.

As noted above, specimen F, for reasons unknown, hardly took up the alizarin red S stain. The alcian blue stain for cartilage was only minimally apparent. Only a slight brown

tinge was noted about the trabeculae and bony collars of the scapulae, mandible and long bones. A very faint tinge of red was observed, with difficulty, in the neural arch ossification centre of the second cervical vertebra.

Specimens B and C cleared much more quickly than those fixed in formalin. The double staining resulted in a vivid contrast of colours making it very easy to differentiate between bone (red) and cartilage (blue) through clear transparent flesh.

Rather than a detailed description of each embryo and fetus examined, observations are presented as Table X, recording the neural arch ossification centres which were visible through a binocular dissection microscope with a magnification of up to 3. Some neural arch ossification centres were barely visible microscopically. The size of an ossification centre could be assessed more accurately using the binocular microscope.

Ossification centres for the vertebral centra were also included for the above embryos and fetuses as a matter of interest. Results are presented in Table XI.

No ossification centres were observed in specimen A. The delicate cartilaginous skeleton was stained a vivid blue and the embryo could nicely be placed in Streeter's Horizon XXII (44-46 days; 25-27 mm). Viscera remained light brown and opaque in nature.

Similarly, specimen D showed no neural arch ossification centres. The blue cartilaginous skeleton was less vivid,

however more mature in form. All other specimens exhibited ossification centres in the neural arches of varying sizes and stages of development.

It is difficult to give a description of the appearance or size of an ossification centre by way of a table. Some centres are present and deeply stained with lizarin d S whereas other centres are barely present and very faintly stained. In each case the ossification centre has been indicated as present, if seen through a microscope.

Nevertheless, in Tables X and XI, an attempt has been made to indicate the ossification centres for the neural arches and centra which are present and in those which appear to be the largest and most fully developed, the "x" is underlined. Where no "x" is present, no ossification centre was observed. Ossification centres on either side of those which are underlined, successively diminish in size.

Almost exclusively, cervical vertebra 4 and thoracic vertebra 9 and 10 appeared as the smallest centres of ossification for the neural arches. As well, cervical 2 neural arch ossification centres were usually longer and thicker than cervical vertebra 1.

Specimens in which ossification has spread as far as lumbar vertebra 5 neural arch, were difficult to assess accurately as some difference in the size of ossification centre appears to exist and yet the difference is slight. For example, specimen J appeared to have larger centres in the upper cervical and upper thoracic region, with those in between being of slightly

lesser caliber. Also, those in the lower thoracic/upper lumbar region were larger than those in the mid-thoracic region, yet smaller than the ossification centres noted above. This degree of variability appeared in specimens H, I, J and K. Therefore, noting Table X, three areas of neural arch ossification seem to be more developed than others; upper cervical, lower cervical/upper thoracic, lower thoracic/upper lumbar.

Specimen H presented bilateral neural arch ossification centres for cervical 3 and partially for cervical 4, shaped like a bowling pin with the narrow end directed posteriorly.

Specimen I exhibited a second ossification centre on the right side, lateral to the neural arch ossification centre for cervical vertebra 7. No other embryo or fetus had this present.

Specimen G presented a blue cartilaginous skeleton with deep red ossification centres. Those of the neural arches were less developed and more easily differentiated. In this specimen, two areas seemed to be further advanced than the others; second cervical vertebra and lower cervical/upper thoracic regions. Of the two, the latter ossification centres were larger. See photographic plates 3 to 6.

Conversely, the vertebral centra were larger and presumably commenced development from one specific area, namely the lower thoracic/upper lumbar region with centres of ossification becoming successively smaller on either side of this region. More ossification centres appeared cranially than caudally to this area. Specifically, the centra of thoracic vertebra 12 appeared to be consistently larger in comparison to adjacent

vertebrae (Table XI).

In specimens H and I, vertebral centra ossification centres appeared as elongated tubes with constricted middles and were situated between T3 and T12 and T5 and T8, respectively. These were suggestive of lateral ossification centres having coalesced.

B HISTOLOGICAL HUMAN SERIAL SECTIONS

In sections of embryos and fetuses, where there was or was not a deposition of calcium in a neural arch ossification centre, a method of assessment was instituted which meant counting the number of slides over which an ossification or pre-ossification centre appeared. Results were subsequently derived by means of a graph which represented the areas of greater or lesser population of cartilage cells in the process of development, prior to ossification.

Specimen F exhibited hyaline cartilage cells in various stages of development which were beautifully outlined in a medium shade of blue-green. A critical examination was undertaken in which 20 vacuolated cartilage cell compartments in Phase III or better of morphological development (Streeter 1949) had to be observed. This number was randomly chosen in this case, in order to set some arbitrary external limit to the growth centre. From the point of initial appearance of these vacuolated cartilage cells in a neural arch ossification centre, the number of sections over which these cells appeared for each centre was noted. When less than 20 vacuolated cells were observed, following the counting, the other side

of the ossification centre was felt to have been reached.

In samples from the RFS collection, no such number was set. The mere presence of vacuolated cartilage cells appeared adequate as most of the sections were of embryos smaller than specimen F. In either case, the end result would simply mean a raising or lowering of the plotted curve.

Results could of course be variable, dependent on the angle at which the centres were sectioned (i.e., sagittal, coronal). That is, if an ossification centre had been cut on the oblique, for example, one could assume that the ossification centre may appear larger or smaller due to its irregular conformity. Again, this was not considered to be of serious consequence as consistency would be maintained for each specimen.

From Tables II to VII it can be seen that neural arch ossification centres appear to be larger and presumably more advanced in some regions. There is a tendency for an elevated curve in the areas of the upper cervical, lower cervical/upper thoracic and lower thoracic/upper lumbar regions. Ossification centres on the right side in specimens examined appear to be generally more advanced in the lower cervical/upper thoracic region than those on the left side. Conversely, those centres on the left appear to be more advanced in the upper cervical region as opposed to those on the right side.

No consistency in growth rate was observed regarding left versus right neural arch ossification centres and a cranial-caudal sequence of development was not obvious.

Correlation between CRL and the number of serial sections over which an ossification centre occurred appeared in the lower thoracic

region of specimens H99 (55 mm) and F (53 mm). On the other hand, no correlation at all was present in any region between specimens H65 (37 mm), H25 (37.5 mm) and H107 (34 mm). Specimen H25 (37.4 mm) clearly indicated a commencement of ossification in two regions; the upper cervical and lower cervical/upper thoracic.

and first two lumbar vertebrae at 17 days and 8-1/4 hours. It was hoped that the specimens from Lot W which were extracted at a later gestational age would have had neural arch ossification centres in a slightly more advanced stage of development.

It will be noted that more neural arch ossification centres were present (almost double the number) on the left side than on the right. In one fetus, #9, ossification centres were slightly tinged on both sides, in the thoracic vertebrae 12 and 13.

By removal of all extraneous surrounding tissue and skeletal framework, visibility of the neural arch ossification centres was excellent although the spinal column assumed a very curved position due to lack of support. Prior to removal of excess tissue, no opaque accumulations of saponified fat were found as claimed by Strong (1925).

In those specimens in which no bone stain was visible in the neural arch ossification centres of cervical vertebra 1, the area was of lighter blue, rough and granular with a clear outer layer (similar to unstained bony collars in long bones). This granular-appearing cartilage represents calcified cartilage which can be stained using Dawson's method (Strong 1925). These same characteristics were apparent in the cervical vertebrae 6 and 7 and thoracic vertebra 1 as well as thoracic vertebrae 10 and 11 in some specimens. Where the laying down of osseous substance had occurred, it was in the form of a red bony collar and appeared constricted in the middle. On either side of the collar, the cartilage was lighter and granular-like and where a slight red tinge was seen, the cartilage was extremely granular.

No ossification centres were seen for the vertebral centra.

B. HISTOLOGICAL SERIAL SECTIONS

Two albino rat specimens, #1 (18.3 mm) and #3 (18.2 mm) were embedded and serially sectioned sagitally at 14μ and 20μ respectively.

Specimen #1 had been processed in chloroform and stained with alizarin red S and alcian blue whereas specimen #3 had been processed in xylene and stained with alcian blue alone. Tissues in specimen #3 were very much more rigid than those in specimen #1 and did not section as easily. As well, at 20μ, the sections were too thick.

Tables VIII and IX are representative graphs produced to demonstrate the results from serial sections of Specimens #1 and #3, in a similar way to human Specimen F and the Dr. RFS collection. That is, vacuolated cartilage cells formed the criteria by which an ossification or pre-ossification centre was assumed to be present.

In some sections of Specimen #3, numerous black fragments were present probably due to poor histological technique. Visibility of cartilage was minimally affected in those sections.

The neural arch of cervical vertebra #1 on the right side of Specimen #1 was the only ossification centre which appeared red. Areas which stained red with alizarin red S in fetus #1, remained mainly white with a slight blue tinge, in fetus #3.

As per Tables VIII and IX ossification was generally more advanced in rat #1 and on the right side, although both #1 and #3 were of similar CRL.

In Table IX, the curve representing rat #3 peaked in three areas; upper cervical, upper thoracic and mid to lower thoracic.

In Table VIII, the curve representing rat #1 could be said to peak in four areas but with a general cranio-caudal decline; upper cervical, lower cervical/upper thoracic, mid to lower thoracic and lumbar.

No conclusive cranio-caudal sequence of development appeared evident.

CHAPTER IV

DISCUSSION

The reliability of external measurements, taken as an estimate of fetal age, is very low. For purposes of comparison, no single method of measurement should be employed (Hamilton, Boyd, 1972). CRL for each individual fetus is particularly affected by the differences in the degree of flexion of one fetus to another.

Prenatal age is actually the time which has elapsed after conception and the date of conceptus cannot be accurately timed in the human (Mall 1910). Precise data of coitus and menstrual history is required to accurately define embryonic age (Streeter 1942, 1945, 1948). Streeter's (1951) development of the Horizons in Human Embryos, in an attempt to determine embryonic age, have been used extensively but unfortunately do not extend into the fetal period, after which onset of bone marrow formation in the humerus has occurred.

Bagnall (1976) suggests expressing embryonic age as "conceptual" rather than "menstrual", whereby ovulation is considered to take place approximately 14 days after the onset of menstruation. A reasonable estimation would be obtained with an error of $\pm$ 1 week (Finnstrom 1972).

Arey (1939) did an analysis of 20,000 calendar records from 1500 individuals and found that no perfect regularity existed in females between the ages of 17 to 49 years. Also, their menstrual cycles varied from 2-3 to 7 weeks. It appears that extensive investigation of this problem has yet to be done.

In this study, CRL was taken merely as the embryo or fetus presented itself, with neither the head nor trunk placed in a position of flexion or extension. In such fixed positions, measurements will always contain some degree of variation and often the fault lies with inadequate definition of technique, statistical data collection and standardization of fetal posture. Notation of CRL was simply to identify and very loosely categorize individual fetuses.

Most material obtained for fetal study has no accompanying history. Clinical history was not available for the material used in this study. Therefore, a gestational age based upon the Last Menstrual Period (LMP) dates was not calculated.

Where pathological conditions existed, one would assume that growth of the fetus had possibly been inhibited or terminated prior to abortion or retrieval of the conceptus. Thus, for a given fetal age, the CRL would presumably be less than that for a normal fetus.

TECHNICAL CONSIDERATIONS

A certain amount of discrepancy is created when formalin fixed fetuses are measured due to swelling or shrinkage of tissues to a certain, although limited extent (Schultz 1919, Scammon and Calkins 1929). O'Rahilly and Meyer (1956) suggest that formalin is a decalcifying agent and Ham (1957) states that mineral deposits in teeth, bone and cartilage are not materially affected by aqueous fixing solutions unless they contain an acid. Where all specimens had been treated in the same way, a certain consistency of measurement could be said to be possible, regarding fetal age.

In view of the current literature, we were not concerned with

calculation of prenatal age but rather the order of appearance of neural arch ossification centres as a "process". The appearance time of these centres was not investigated.

Regarding the evolution of the notochord and the principle of rearrangement of the sclerotomes and the differing opinions put forward for both, no conclusions were drawn from this project. Future work could well contemplate a comprehensive and extensive histological investigation using a large sample of human embryos and fetuses.

In a sample of 40 fresh human specimens, Noback (1944) was the forerunner in the use of simultaneous alizarin red S and KOH staining and clearing for demonstration of the "in situ" skeleton. From his investigation he defined four morphological types of developing prenatal bone, stating that alizarin red S was specific to bone (Cameron 1930) and a differential staining affinity of "alizarin" to bone existed in sites of recent ossification.

A perusal of the literature on stain technique reveals that disagreement has existed as to the specificity of alizarin red S to calcium salts. Recently, however, the alizarin red S technique has been modified by Watson (1977) to demonstrate prenatal ossification. The Watson method is the one employed in this study.

Except for the use of alcian blue for cartilage, Noback's (1944) procedure was similar to that used in the present study for fresh and fresh frozen material (rat and human specimens B and C). The stained fresh specimens retained their colours of red and blue effectively, even though they were stored in glycerin for over one year.

Material fixed and stored in 10% formalin took longer to process in every aspect of the procedure (Table 1) and showed a definite

leaching out of colour, particularly that of alcian blue. Nevertheless, using trypsin as a clearing agent as indicated by Watson (1977) the procedure appeared to be superior to those which reported inadequate clearing of tissues, especially in larger vertebrates.

Fresh rat fetuses stained well with alcian blue and alizarin red S providing an excellent contrast of colours.

Newborn albino test rats also processed extremely well although they had been fixed and stored for a lengthy time in a 10% formalin solution. Most surrounding flesh and skeletal framework had been removed with the cartilaginous and bony axial skeleton exposed to the stains. The surrounding tissue that remained became clear and transparent and for reasons unknown, the vivid contrast of colours did not fade under similar circumstances when stored in glycerin for over one year. These specimens, however, had been allowed to progress to full term without interruption whereas the human specimens might well have had associated pathology.

CRL would not be a consideration regarding the difference in retention of stain, in that both test rats measured out at 56 mm, larger than those specimens of Dingerkus and Uhler (1977).

One of the newborn test rats was subjected to a reversal of technique in order to stain the cartilage and bone a deeper color. Subsequently, tissues cleared well and the specimens provided an excellent contrast of colors, namely a deep blue and red for cartilage and bone. No overstaining or maceration of tissues was apparent with simultaneous staining and clearing in KOH solution. On the other hand, when the process was reversed on fresh rat fetuses (#'s 2, 4, 5 and 6) discoloration of surrounding tissues occurred, although visibility was

not obstructed.

The object of a further series of experiments would be to consider varying the fixative, temperature of the solutions, time of immersion, brand and strength of the stain with the variability due to size of fetus.

No obvious detrimental effect was apparent in human specimen F and rat specimens 1 and 2 when the material was stained and sectioned for microscopic study following Watson's (1977) procedure. Rat specimens processed in Chloroform rather than xylene were much easier to section.

Although one rat specimen and most of the RFS collection had been sectioned at 20 u, this investigation determined 12 to 14 u as much more satisfactory. Less destruction and crinkling of the paraffin ribbon occurred.

If rat specimens were to be studied postnatally, madder feeding could be considered although with a rather uncertain feeding procedure. Injections of 200 mg/kg per week appear satisfactory (Lillie 1965). Prenatally, the placental barrier discards all notions for use of the above procedure.

THEORETICAL CONSIDERATIONS

Bagnall (1977) defines a centre of ossification, using the radiographic method, as a change in radio-translucency within the cartilage mass. This definition does not distinguish between true bone and calcified cartilage or endochondral and intramembranous ossification.

Meyer and O'Rahilly (1958) in histological examination used vascular invasion of cartilage masses as a criterion for the onset of

endochondral ossification.

Ham (1957) defines centre of ossification as that state "when the osteogenic cells, the osteoblasts and the capillaries of the periosteal bud reach the interior of the midsection of the cartilage model".

Meyer and O'Rahilly (1958) showed that alizarin red does not demonstrate the onset of endochondral ossification but rather the laying down of the intramembranous periosteal collar.

Gardner (1971) suggests that the laying down of a periosteal collar is an indication of impending endochondral ossification, forming slightly before or at the same time. Thus, one could contend that regardless of the type of osseous deposit, a centre of ossification as defined by Ham (1957) was present or imminent.

Thus, in albino rat specimens, stained with alcian blue and alizarin red (Table XII) the appearance of a red collar-like structure in the lateral masses of the 1st cervical vertebra might be a primary osseous shelf which would presumably coincide with a centre of ossification or appear slightly in advance of a centre of ossification. Where this occurs, only the first cervical neural arch, one could assume that ossification commences in that area.

This study concurs with Gardner (1971) in that ossification can be noted histologically slightly earlier than with clearing of whole specimens (Human specimen F). Histological methods largely avoid the leaching of bone salts from the tissues and this method of examination was especially useful in determining whether or not a particular pre-ossification or ossification centre was present (Tables II to VII).

Histologically, hypertrophy of cartilage cells suggests a potential

or actual secretion of phosphatase with subsequent calcification of the organic intercellular substance of the cartilage, a stage of osteogenesis (Streeter 1949). At about the same time, the osteogenic cells of the perichondrium have differentiated into osteoblasts and have laid down a thin layer of bone intercellular substance (periosteal substance) (Ham 1957). The latter constitutes the bony collar or primary osseous shell in tubular bones (Streeter 1949) preceding bone-marrow formation.

In rat specimens, Bloom and co-workers (1940) have shown that in the humerus, ulna and radius the deposition of calcium takes place slightly in advance of the penetration of blood vessels.

In view of the above information, ossification in this study was considered as that time when alizarin red S stain is obvious microscopically. Furthermore, vacuolization of cartilage cells seen histologically, was considered as that stage when calcification was present prior to and during ossification.

Disagreement surrounds the sequence of development of the neural arch ossification centres in the human, with most textbooks reporting that ossification of the neural arches occurs in a cranio-caudal direction (W and W 1973, Davis and Dobbing 1974). That is, they suggest that neural arch ossification centres first appear in the upper cervical vertebrae and progress successively to lower centres.

From Table X it can be seen that a consistent pattern of cranio-caudal development of neural arch ossification is not apparent. Mall (1906) made the assumption that the largest centre to be seen must be the first centre to have ossified. As most specimens had larger neural arch ossification centres in the lower cervical/upper thoracic region, suggests that these centres were the first to ossify.

In the upper cervical region, the neural arch ossification centres of vertebra 2 displayed almost consistently a further stage of ossification than its neighbors although as a region, it was not as advanced as the lower cervical/upper thoracic region.

In accordance with Meyer and O'Rahilly (1958) histological examination was conducted in addition to in-toto staining in order to more accurately recognize the advent of the onset of endochondral ossification. Tables II to VI illustrate the progression in development of ossification of the various regions. Although the degree of development is not the same for both sides, it is obvious that a cranio-caudal sequence has not occurred. On the right side, four cases show more advancement of ossification in the lower cervical/upper thoracic region than in the upper cervical or lower thoracic/upper lumbar regions. On the left side, with the exception of one case in the upper cervical region, two cases show more advancement of ossification in the lower cervical/upper thoracic region and three cases show more advancement of ossification in the upper cervical region although a minimal difference exists in Tables V and VI.

More data would be required to substantiate the view that ossification of neural arches commences in the lower cervical/upper thoracic regions although these tables suggest this as a possibility.

In any case, from Table X and Tables II to VII, and in agreement with Bagnall (1977) it can be seen that although no exact pattern for the appearance of the neural arch ossification centres emerges, three groups of centres appear. A first group appears in the lower cervical/upper thoracic region quickly followed by a second group of centres in the upper cervical region, cervical vertebra 2 neural arch being the

largest centre. The remaining cervical neural arches ossify prior to the appearance of a third group in the lower thoracic/upper lumbar region.

In Table VII (Specimen H99 55 mm) in which only the lower torso was available for study, it could be suggested that a complete graph would be similar in conformity to Table II (Specimen F 53 mm). Advancement of neural arch ossification and CRL are similar in both cases.

Teissandier (1944) concluded that cervical arches 2 and 7, thoracic arches 1 and 12, and lumbar arch 1 are the first of the neural arches to ossify.

Noback & Robertson (1951) concluded that a pattern of ossification similar to that suggested by Teissandier (1944) may occur.

Mall (1906) concluded in his description of two human specimens that ossification of the vertebral neural arches commenced on the 57th day in the second (cervical 2) and 8th (thoracic 1) arches. On the next day (58th day) ossification had spread from these two points to include cervical 1, 2, 3 and thoracic 1 to 4 on the left side; cervical 1, 2 and cervical 7 to thoracic 3 on the right side. By the 64th day, ossification had spread to lumbar 3. He states that the lower vertebrae vary in their appearance, similar to the situation in ribs. He makes no mention of a third group commencing ossification in the lower thoracic/upper lumbar region. However, he stresses that the cervical 2 neural arch is the largest in the cervical region and thoracic 1 or 2 or both, are largest in the upper thoracic region.

Bagnall (1977) suggests a pattern of ossification originating from three regions: lower cervical/upper thoracic to upper cervical following which the remaining cervical arches ossify, to lower thoracic/upper lumbar regions. He further suggests that ossification spreads cranially from the third centre to meet those spreading caudally from the upper thoracic region and that the lower lumbar and sacral regions develop

more slowly but in a caudal direction.

Bagnall (1977) presents six specimens, ranging in CRL from 47.5 mm to 61.0 mm in which ossification of neural arches was apparent only in the lower cervical, upper thoracic regions. Thoracic vertebra I neural arch was consistently included in all six specimens. In three specimens, ranging in CRL from 55.0 mm to 59.0 mm, two groups of centres had appeared in the lower cervical/upper thoracic and upper cervical regions. Cervical vertebrae 2 ossification centre was present in all three specimens. As well, seven specimens ranging in CRL from 55.0 mm to 64 mm, presented a third group separate to the other two groups, in the lower thoracic/upper lumbar region.

The above data presented by Bagnall (1976) indicate that the sequence of development of neural arch ossification is not in a craniocaudal direction.

The results of the present study from a total of 15 selected human fetuses and embryos, tends to support the conclusions drawn by Teissandier (1944) and Bagnall (1977). Although only two specimens (Specimens C & G) demonstrate a division of neural arch ossification in the mid-thoracic and mid-cervical regions respectively, the stage of development of ossification (as per Mall 1906) indicates that ossification commences in two basic regions, namely, the cervical and lower thoracic/upper lumbar. It is also suggested that the order of appearance of neural arch ossification centres is such that centres in the lower cervical/upper thoracic region appear first, followed by centres in the upper cervical and lower thoracic/upper lumbar regions. See Tables II to VII and Table X. No centres of ossification were seen in Specimens A (27 mm) and D (44 mm).

The vertebral centra, on the other hand, as reported in current textbooks (W & W 1973) appear to commence ossification in the lower thoracic/upper lumbar region (Table XI). In this study the largest centres of ossification occurred in thoracic vertebrae 10, 11, 12 and lumbar 1 and had advanced in both cranial and caudal directions, however, more quickly in the cranial than caudal direction. No individual vertebra could be identified as the first to have ossified. No conclusions were drawn as to whether or not ossification of the centra preceded ossification of the neural arches, and no ossification centres were seen in cervical 1 centra. This concurs with the literature in that ossification takes place in cervical 1 postnatally.

As seen in Table X, an absence of one of a pair of neural arch ossification centres was not uncommon and Noback and Robertson (1951) reported similar findings. These centres appeared to be in areas where ossification would start and one can only assume the centra would have developed had the fetus been allowed to progress. Christie (1949) maintained assymetry was insignificant and O'Rahilly and Meyer (1956) considered an ossification centre present whether uni or bilaterally situated.

When each of a pair of ossification centres was considered individually, one would occasionally appear to be at a different stage of development. This observation was made by noting differences in size and degree of staining in whole specimens, as well as size and degree of staining in whole specimens, as well as size and degree of vacuolization of cartilage cells histologically.

The appearance time of ossification centres for the neural arches has never been definitely settled mainly due to indefinite embryonic

aging and a lack of large numbers of specimens. Bagnall (1977) suggests that ossification may be related to fetal head, upper and lower limb movements.

In the present study of albino rat fetuses, it is suggested that further investigation would be necessary, with greater variability in fetal age, in order to develop a sequence of neural arch ossification. Table XII illustrates the stages of ossification which had taken place prior to extraction of the conceptus.

Strong (1925) reports that ossification of the vertebral arches occurs in the arches of the cervical vertebrae, especially in the more anterior and the first two lumbar vertebrae at 17 days + 8-1/4 hours. We have noted a similarity to his findings in only one case, Specimen 9 (18.8 mm) and even then, the lightly stained ossification centre was minimally visible in thoracic 12 and 13. Whether or not this is conclusive of a second group of ossification centres occurring, cannot be established as more data would be required with a wider age variability.

Strong (1925) also mentions the occurrence of "a second centre somewhat dorsal to this in all of the cervical vertebrae except the first two and in the first thoracic vertebrae of specimens". He notes that there is a tendency for the time of first ossification to be successively later in more caudal vertebrae and the last vertebra to ossify is the most caudal of the caudal series.

In the specimens studied, the cervical arch I ossification centres were similar to periosteal collars. However, histologically endochondral ossification was apparent. Tables VIII and IX indicate the degree of ossification in the neural arches, according to the stage of vacuolization of cartilage cells.

The results obtained from albino rats suggest that a cranio-caudal pattern of neural arch ossification is not apparent and that no sequence of development can be established.

Both rat specimens in Tables VIII and IX were at different stages of development, although of similar CRL. Appearance times of ossification centres related to fetal age and CRL could not be established from so small a sample.

CONCLUSIONS

Commencement of ossification in the vertebral neural arches was considered as that time when alizarin red S stain became microscopically visible. The degree of accumulation of the orange-red precipitate was used as a measure for determining which centre had begun to ossify first. Histologically, the morphological changes of cartilage cells were used as criteria for determination of whether or not a site of ossification was in the making.

From our findings, it appears evident that human neural arch ossification begins in two general regions; cervical and lower thoracic/upper lumbar. Furthermore, it appears possible that centres first appear in the lower cervical/upper thoracic regions, rapidly followed by centres in the upper cervical then lower thoracic/upper lumbar regions. Ossification does not proceed in a strict cranio-caudal sequence of development, as reported in current textbooks. That is, although a general cranio-caudal pattern of development exists in that the fifth sacral vertebra is the last to ossify, there are regional differences.

Ossification of the vertebral centra commences in the lower thoracic/upper lumbar regions and spreads in both cranial and caudal directions, more quickly in the former. This observation is in agreement with current textbooks.

TABLES

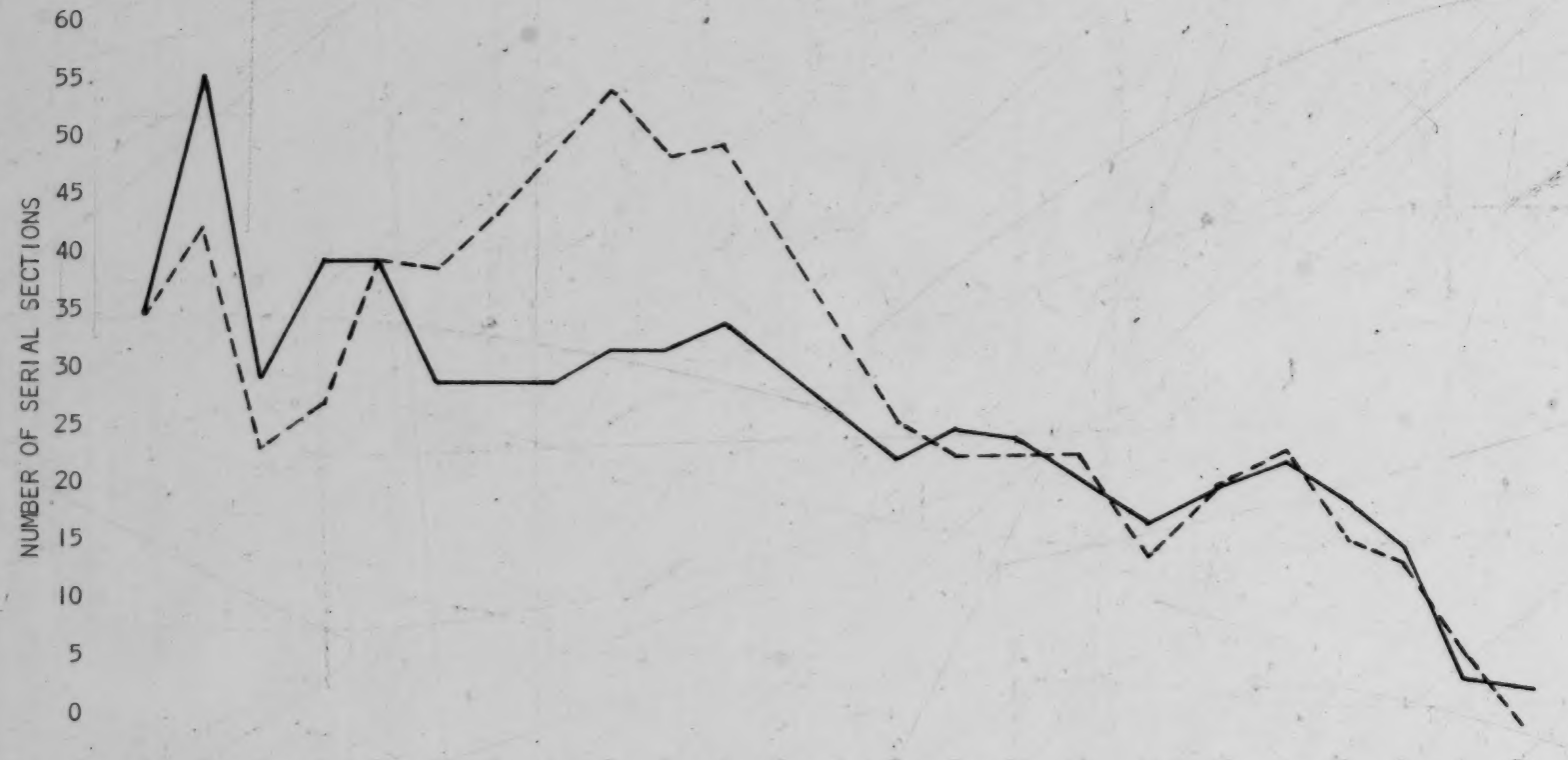
TABLE I

INDIVIDUAL TREATMENT OF TEN HUMAN EMBRYOS THROUGHOUT STAINING PROCEDURE

Specimen No. CRL	Intact	Initial Evisceration	Subsequent Evisceration	Time in Alcian Blue	Time in Alizarin Red S	Time of Final Clearing and No. of Changes	Skin Perforations Abdominal Slit
FORMALIN FIXED							
A 38 mm	x			24 hrs.	30 mins.	27 days x 10 changes	
D 44 mm			After 24 hrs. in Alcian Bl. stain	24 hrs.	30 mins.	26 days x 12	x
F 53 mm			Final clearing	3 days x 3 changes	See text	Approx. 14 days See text	x
G 57 mm			Final clearing	48 hrs.	45 mins.	28 days x 12	x
H 62 mm			Final clear. Skinned & fleshed out initial cl.	48 hrs.	40 mins.	36 days x 8	x
I 68 mm		x Skin & fleshed out	Further skin & flesh. out	48 hrs.	40 mins.	35 days x 8	
J 74 mm		x		48 hrs.	45 mins.	26 days x 10	
K 76 mm	x			48 hrs.	45 mins.	28 days x 10	
FRESH FROZEN							
B 50-55 mm	See text	x		24 hrs.	4 hrs.	17 days x 4	
C 55-60 mm	See text	x		24 hrs.	4 hrs.	17 days x 4	

TABLE II

GRAPHIC REPRESENTATION OF HISTOGENESIS OF CARTILAGE IN NEURAL ARCHES



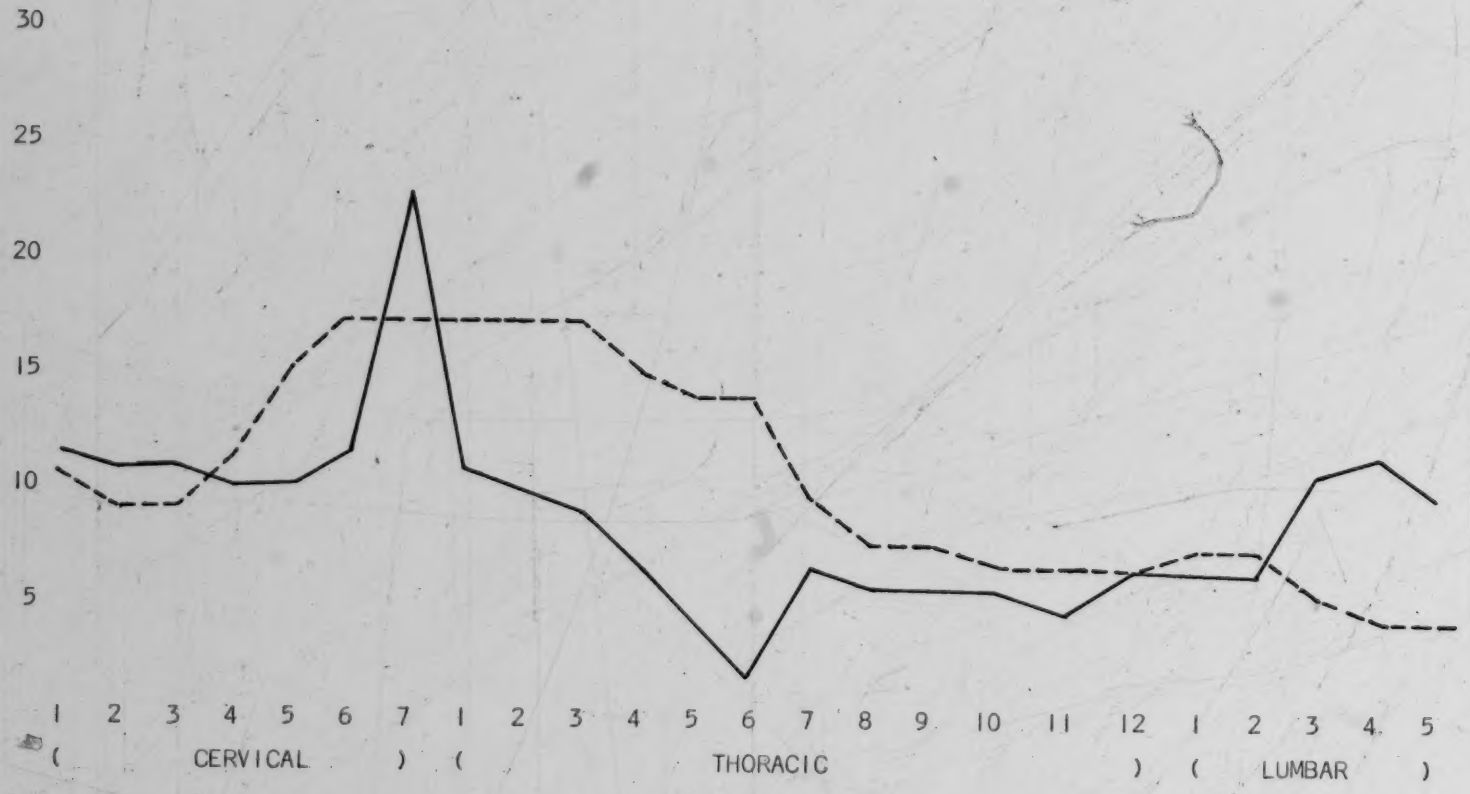
Specimen F; 53 mm; Coronal Section @ 12 u

---- Right

____ Left

TABLE III

GRAPHIC REPRESENTATION OF HISTOGENESIS OF CARTILAGE IN NEURAL ARCHES



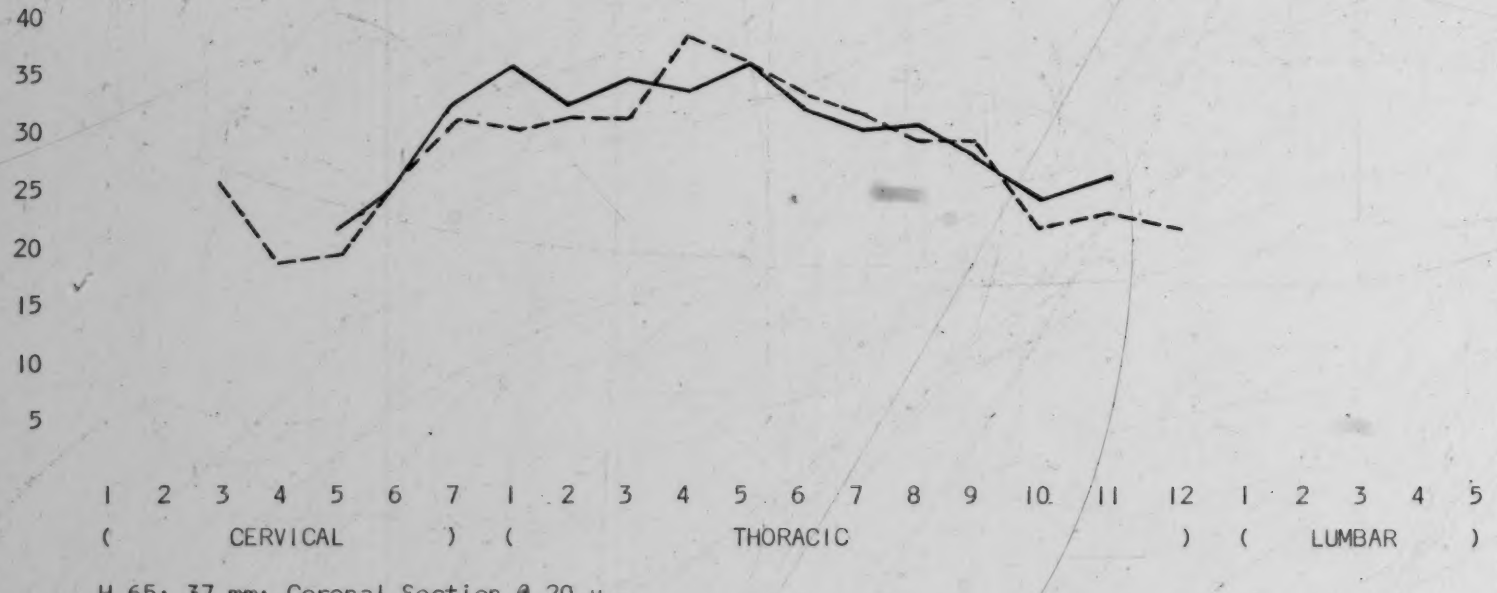
H 107; 34 mm; Sagittal Section @ 20 u

---- Right

____ Left

TABLE IV

GRAPHIC REPRESENTATION OF HISTOGENESIS OF CARTILAGE IN NEURAL ARCHES



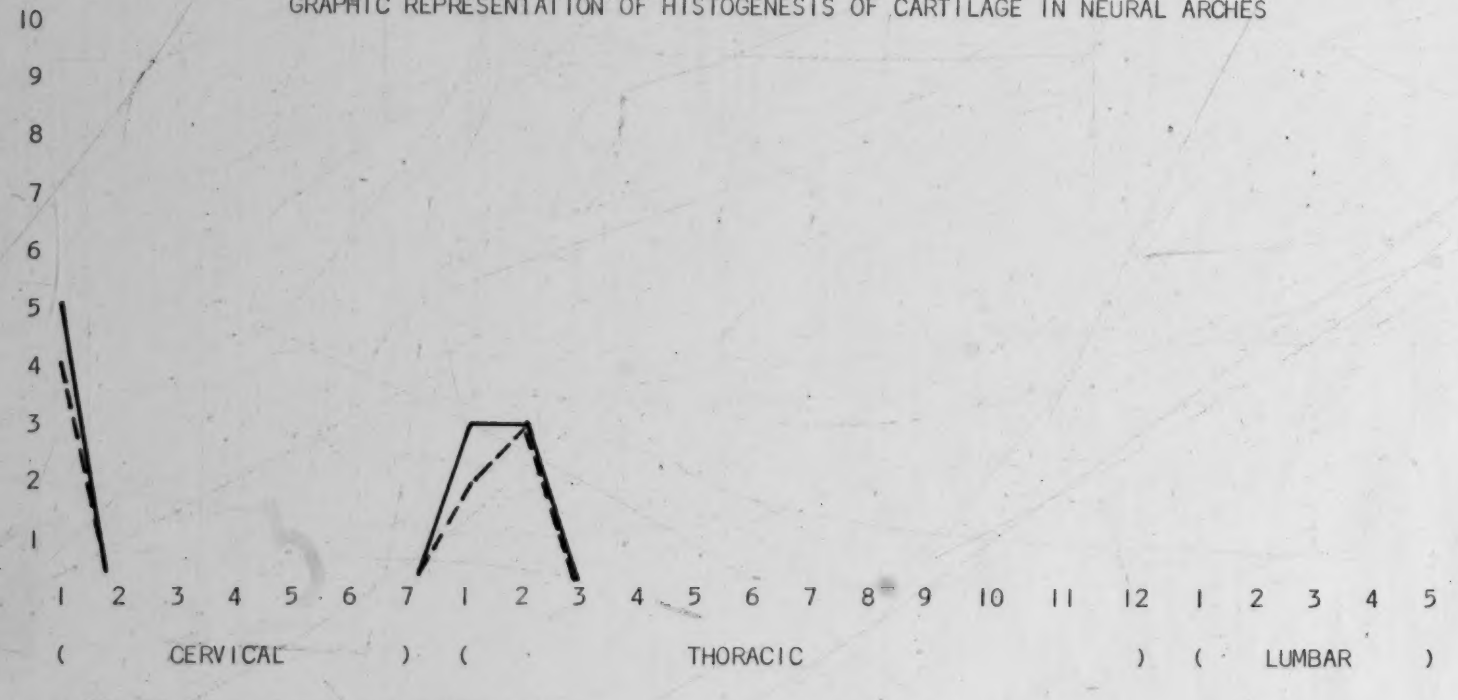
H 65; 37 mm; Coronal Section @ 20 u

---- Right

____ Left

TABLE V

GRAPHIC REPRESENTATION OF HISTOGENESIS OF CARTILAGE IN NEURAL ARCHES



H 25; 37.5 mm; Coronal Section @ 25 u

---- Right

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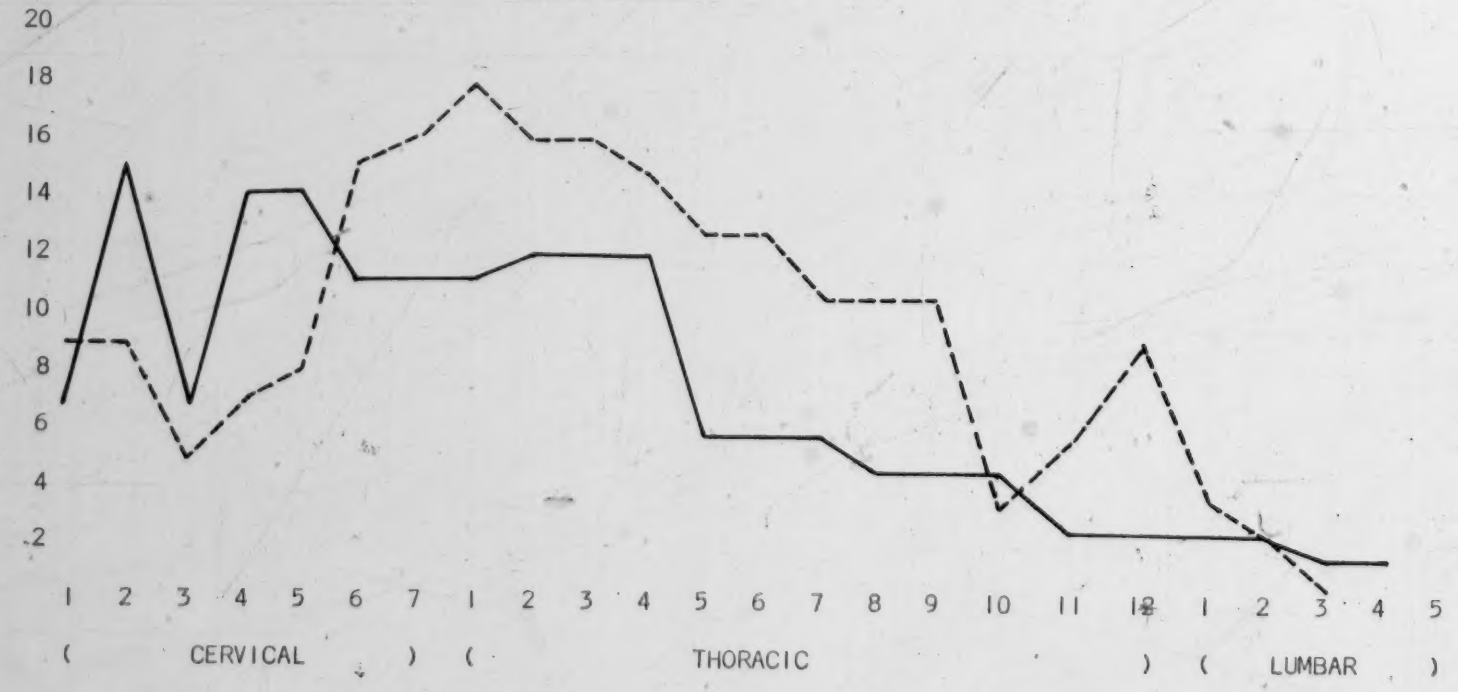
TABLE VI

GRAPHIC REPRESENTATION OF HISTOGENESIS OF CARTILAGE IN NEURAL ARCHES



H 70; 42 mm; Coronal Section @ 20 u

---- Right

____ Left

TABLE VII

GRAPHIC REPRESENTATION OF HISTOGENESIS OF CARTILAGE IN NEURAL ARCHES

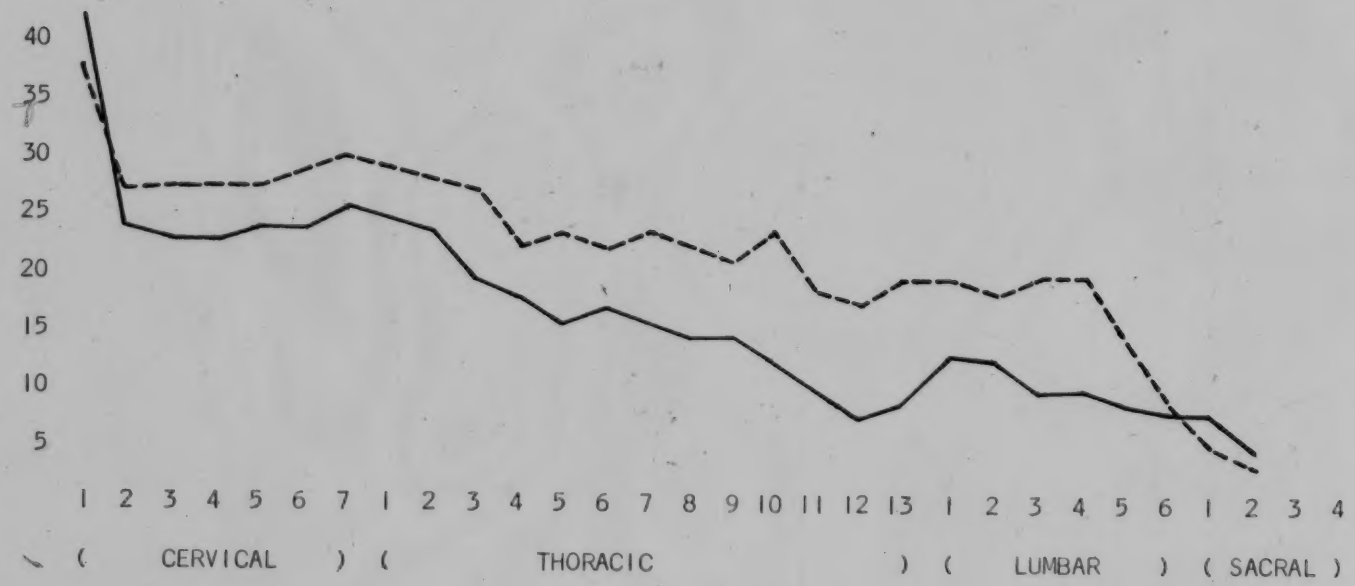
H 99; 55 mm; Coronal Section @ 34 u

---- Right

____ Left

TABLE VIII

GRAPHIC REPRESENTATION OF HISTOGENESIS OF CARTILAGE IN NEURAL ARCHES



Specimen #1; Albino Rat; 18.3 mm; Sagittal Section @ 14 u

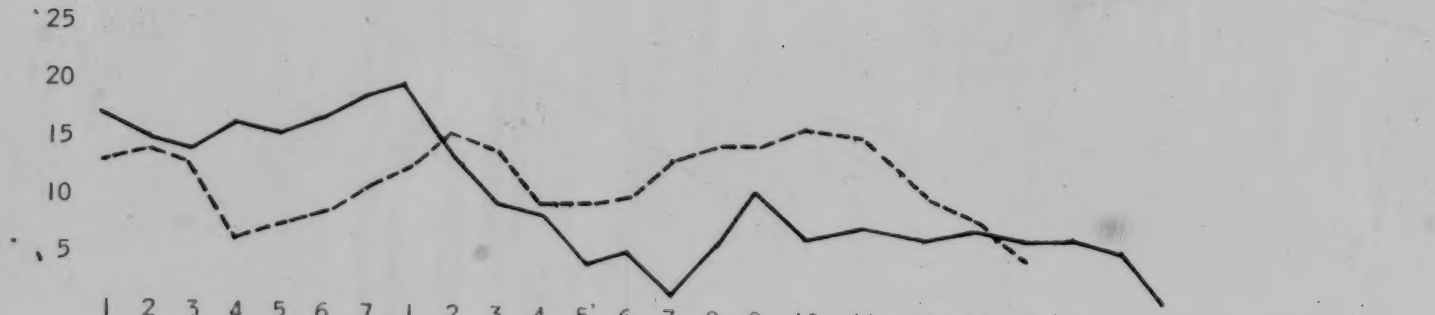
Alcian Blue, Alizarin Red S

----Right

____Left

TABLE IX

GRAPHIC REPRESENTATION OF HISTOGENESIS OF CARTILAGE IN NEURAL ARCHES



Specimen #3; Albino Rat; 18.2 mm; Coronal Section @ 20 u

----Right

____Left

TABLE X

HUMAN SPECIMENS

SPECIMEN	A		B		C		D		G		H		I		J		K	
CRL (mm)	27		50-55		55-60		44		57		64		67.5		74		76	
SIDE	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
CI			×	x̲	×	×			×	×	×	×	×	×	×	×	×	×
2			x̲	×	x̲	×			x̲	x̲	x̲	x̲	x̲	x̲	x̲	x̲	x̲	x̲
3			×	×	×	×			×	×	×	×	×	×	x̲	x̲	x̲	x̲
4			×	×	×	×				×	×	×	×	×	×	×	×	×
5			×	×	×	×			×	×	×	×	×	×	×	×	×	×
6			×	×	×	×			×	×	x̲	×	×	×	×	×	×	×
7			×	×	x̲	×			×	x̲	x̲	x̲	x̲	x̲	x̲	x̲	x̲	×
TI			x̲	x̲	x̲	x̲			x̲	x̲	×	×	x̲	x̲	x̲	x̲	×	x̲
2			×	x̲	x̲	×			x̲	x̲	×	×	×	×	×	×	×	x̲
3			×	×	×	×			×	×	×	×	×	×	×	×	×	×
4			×	×	×	×			×	×	×	×	×	×	×	×	×	×
5				×	×	×			×	×	×	×	×	×	×	×	×	×
6					×				×	×	×	×	×	×	×	×	×	×
7									×	×	×	×	×	×	×	×	×	×
8									×	×	×	×	×	×	×	×	×	×
9									×	×	×	×	×	×	×	×	×	×
10					×	×			×	×	×	×	×	×	×	×	×	×
11					x̲	×			×	×	×	×	×	×	×	×	×	×
12					x̲	x̲				x̲	x̲	x̲	x̲	x̲	×	x̲	×	x̲
LI					×						x̲	x̲	x̲	x̲	x̲	x̲	x̲	×
2											×	×	×	×	×	×	×	×
3											×	×	×	×	×	×		
4											×	×	×	×	×	×		
5											×		×	×	×	×		
I																		
2																		
3																		
4																		

Sequence for the appearance of the vertebral neural arch ossification centres:

C = cervical; T = thoracic; L = lumbar; S = sacral; x = neural arch oss. centre; R = right; L = left; x̲ = largest ossification centre in region.

TABLE XI

HUMAN SPECIMENS

SPECIMEN	A	B	C	D	G	H	I	J	K
CRL (mm)	27	50-55	55-60	44	57	64	67.5	74	76
C1									
2									
3									
4									
5						×			
6						×	×	×	
7						×	×	×	
T1						×	×	×	×
2					×	×	×	×	×
3					×	×	×	×	×
4					×	×	×	×	×
5					×	×	×	×	×
6					×	×	×	×	×
7					×	×	×	×	×
8					×	×	×	×	×
9			×		×	×	×	×	×
10		×	×		×	×	×	×	×
11		×	×		×	×	×	×	×
12		×	×		×	×	×	×	×
L1		×	×		×	×	×	×	×
2		×			×	×	×	×	×
3					×	×	×	×	×
4					×	×	×	×	×
5					×	×	×	×	
S1						×	×	×	
2						×	×	×	
3						×		×	
4									
5									

Sequence for the appearance of the vertebral centra ossification centres:

C = cervical; T = thoracic; L = lumbar; S = sacral; × = centra oss. centre; × = largest ossification centre in region indicated.

TABLE XII

RAT SPECIMENS

STUDY #	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15		16		17		18	
LENGTH (mm)	18.3		19.2		18.2		17.5		16.9		18.2		17.6		17.7		18.8		18.1		17.9		18.4		19.2		18.2		18.7		16.8		16.6		17	
SIDE	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
CI			x	x					x	x		x		x	x	x		0	0	0		x		x		x	x	x	0	x	0	x				x	
2																																				
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4																																				
5																																				
6																																				
SI																																				
2																																				
3																																				
4																																				

Sequence for the appearance of the vertebral neural arch ossification centres:
C = cervical; T = thoracic; L = lumbar; S = sacral; L = left; R = right; x = definite stain obvious;
0 = tinged; barely noticeable extremely granular collar

FIGURES

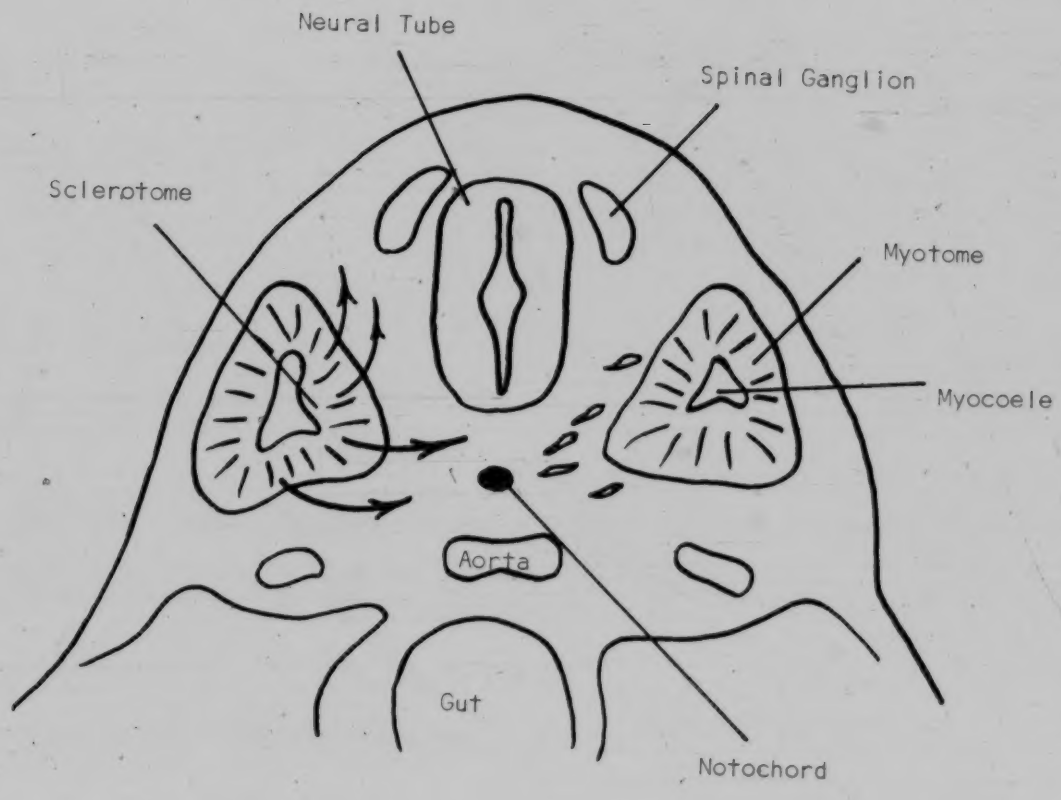


Figure 1. Migration of Sclerotomic Cells.

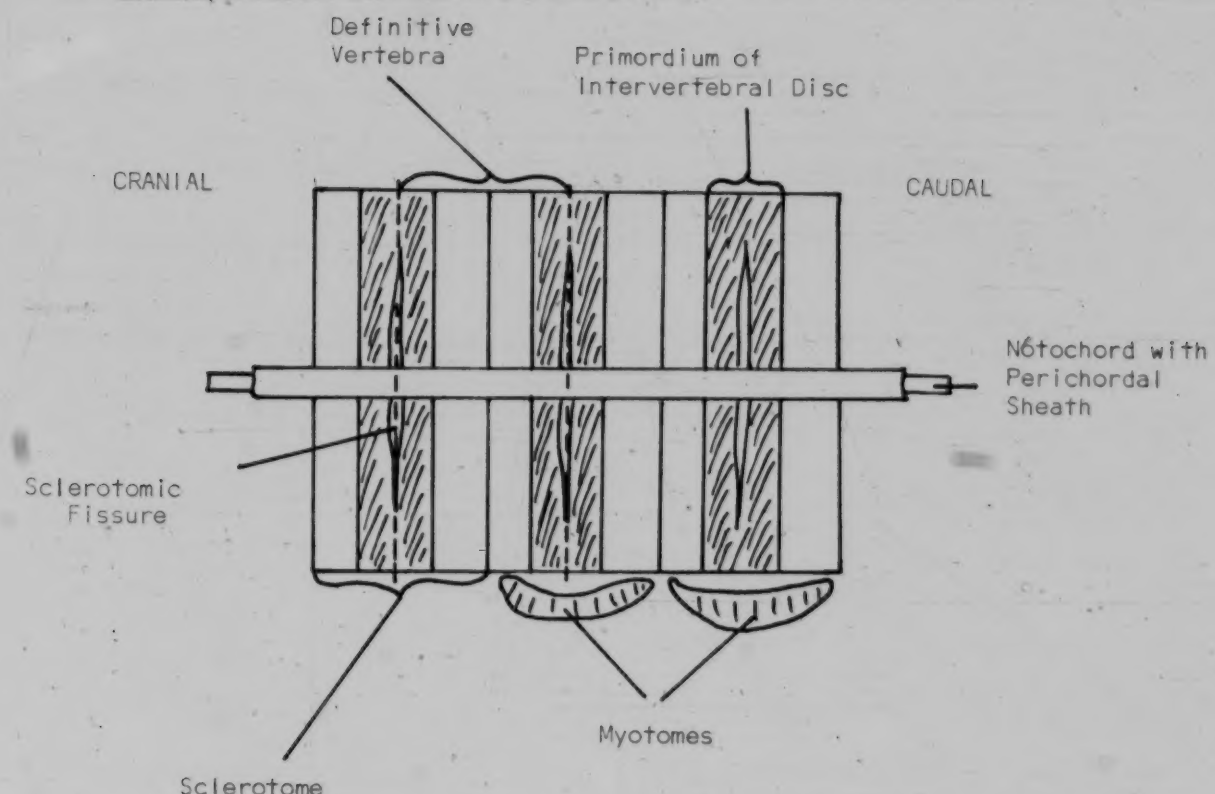


Figure 2. Diagrammatic representation showing the manner in which adjoining segments form the vertebral body. After Gray's Anatomy.

PHOTOGRAPHIC PLATES

Plate 1. Specimen G: to illustrate the deep red ossification centres and bright blue cartilaginous skeleton using the Watson (1977) technique.

Plate 2. Test rat: to illustrate the granular-like appearance of calcified cartilage adjacent to the sites of ossification.

Plate 3: Specimen G: View of left neural arches to illustrate the degree of concentration of Alizarin Red S stain in two regions with a lack of stain in the 4th neural arch.

Plate 4: Specimen G: View of right neural arches to illustrate the degree of concentration of Alizarin Red S stain in two regions but continuous through all cervical and upper thoracic arches.

Plate 5: Specimen G: Closeup of left neural arches. Note that ossification appears more advanced in the second cervical and lower cervical/upper thoracic regions.

Plate 6: Specimen G: Closeup of right neural arches. Ossification is advanced in regions similar to Plate 5; the 4th cervical neural arch shows an ossification centre present.

Plate 7: Specimen B: Neural arches of the right side showing more advancement of ossification in the first thoracic vertebra (arrow).

Plate 8: Specimen B: Neural arches of the left side showing more advancement of ossification in the first thoracic vertebra (arrow). The first and second cervical neural arches demonstrate small centres of ossification in comparison.

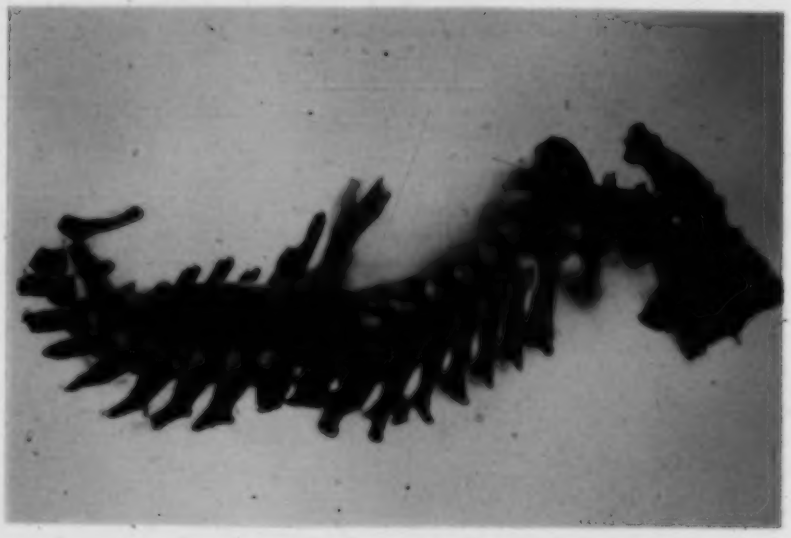


Plate 9: Specimen C: Right neural arch centres commencing ossification in thoracic vertebrae 10, 11 and 12 (arrows). Note the commencement of ossification in the centra of the lower thoracic/upper lumbar vertebrae.

Plate 10: Specimen G: Commencement of ossification in the centra of thoracic vertebrae 5 through 12, lumbar vertebrae 1 through 5 and sacral vertebrae 1 through 3. Note that centres of ossification are larger in the lower thoracic/upper lumbar region (arrows).

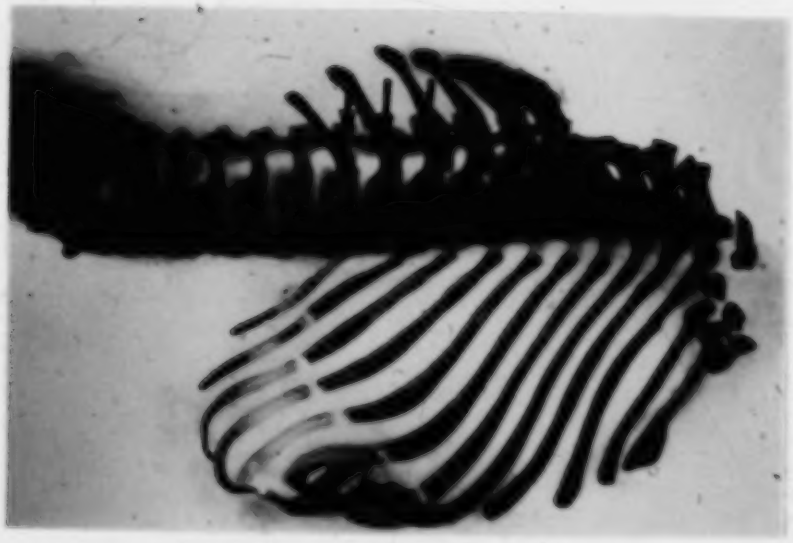
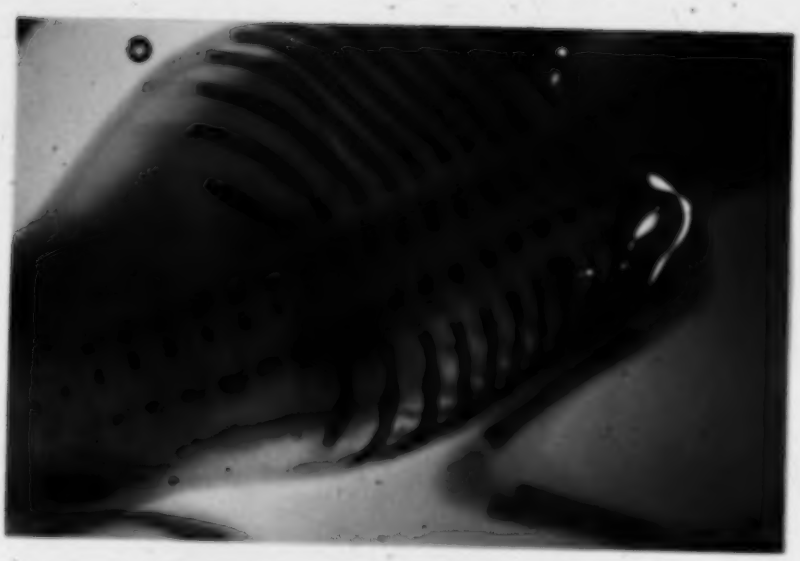


Plate 11: Specimen K: Left side; ossification has advanced in neural arches from the first cervical to the fifth lumbar vertebrae. Smaller centres appear in the mid-cervical, mid-thoracic and lower lumbar regions.

Plate 12: Specimen K: Right side; similar situation to Plate 11. Note the larger vertebral centra in the lower thoracic and upper lumbar regions.



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